

Investigations of the cancer therapeutic and protective effects of warfarin-mediated inhibition of the receptor tyrosine kinase AXL

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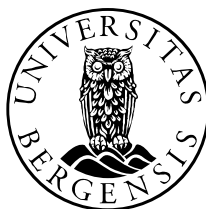
Title: Investigations of the cancer therapeutic and protective effects of warfarin-mediated inhibition of the receptor tyrosine kinase AXL

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Scientific environment

This work was performed at the Department of Biomedicine, Centre for Cancer Biomarkers, Faculty of Medicine, University of Bergen, during the period of 2011-2017. The work has been conducted with Professor James B. Lorens as main supervisor and Professor Oddbjørn Straume as co-supervisor. From August 2012-December 2012, I had a predoctoral fellowship at UT Southwestern, Dallas, Texas, USA, in the research lab of Dr. Rolf Brekken. The Faculty of Medicine provided financial support for the PhD-fellowship. The experimental work was supported by the Faculty of Medicine, Helse-Vest, and the Research Council of Norway through its Centers of excellences funding scheme.



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Abbreviations

| | |
|------------|---|
| AC | Apoptotic cells |
| ACC | Acinar cell carcinomas |
| AML | Acute myeloid leukemia |
| ATP | Adenosine triphosphate |
| BAD | Bcl- 2 associated death promoter |
| Bcl-2 | B cell lymphoma 2 |
| Bcl-XL | B cell lymphoma extra large |
| BCSC | Breast cancer stem cells |
| BRAF | B-Raf proto-oncogene serine/threonine kinase |
| BRCA2 | Breast cancer 2 |
| C1-TEN | C1 domain containing phosphatase and TENsin homologue |
| Cbl | Casitas B-lineage lymphoma |
| CDKN2A | Cyclin dependent kinase inhibitor 2A |
| CI | Confidence Interval |
| CLL | Chronic lymphatic leukemia |
| CRN | Cancer registry of Norway |
| CSC | Cancer stem cell |
| DKK3 | Dickkopf related protein 3 |
| DPC4 | Deleted in pancreas cancer 4 |
| E-cadherin | Epithelial cadherin |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial to mesenchymal transition |
| ERK | Extracellular signal regulated kinase |

| | |
|--------------------------------|---|
| FGF | Fibroblast growth factor |
| GAS6 | Growth arrest spesific 6 |
| GEMM | Genetically engineered mouse models |
| GGCX | Gamma-glutamyl carboxylase |
| GSK3 | Glycogen synthase kinase 3 |
| HGF | Hepatocyte growth factor |
| HIF | Hypoxia induced transcription factor |
| ICD | International classification of diseases |
| ICD-O-3 | International classification of diseases for oncology |
| IFNAR | Interferon α/β receptor |
| IG | Immune globuline |
| IGF | Insulin like growth factor |
| IPMN | Intraductal papillary mucinous neoplasm |
| IRR | Incidence rate ratio |
| KO | Knockout |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LMWH | Low molecular weight heparin |
| MAPK | Mitogen activated protein kinase |
| MET | Mesenchymal to epithelial transition |
| miR | MicroRNA |
| MK | Menaquinone |
| MMP | Matrix metallo- proteinases |
| N-cadherin | Neural Cadherin |
| NF-κB | Nuclear factor κ B |
| NK cells | Natural Killer cells |
| NOAC | Non-vitamin k anticoagulant |
| NorPD | Norwegian Prescription database |

| | |
|-------------------------------|---------------------------------------|
| PAK1 | P-21 activated kinase |
| PanIN | Pancreatic intraepithelial neoplasias |
| PI3K | Phosphoinositol 3 OH kinase |
| PD1 | Programmed Death Protein 1 |
| PDAC | Pancreatic ductal adenocarcinoma |
| PNET | Pancreatic neuroendocrine tumors |
| PtdSer | Phosphatidylserine |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| sAXL | Soluble AXL |
| SMAD4 | SMAD family member no 4. |
| SOCS | Suppressor of cytokine signaling |
| Sp | specificity protein |
| TAM | Tyro, AXL, Mer |
| TERT | Telomerase reverse transcriptase |
| TLR | Toll-like receptor |
| TGF-β | Transforming growth factor β |
| TNFα | Tumor necrosis factor α |
| TNM | Tumor, Node, Metastasis |
| VEGF-A | Vascular endothelial growth factor A |
| VHL | Von Hippen Lindau protein |
| VKOR | Vitamin K epoxide reductase |
| VKDP | Vitamin K dependent proteins |
| VSMC | Vascular smooth muscle cells |
| ZEB | Zinc finger E-box binding homeobox |

Abstract

Cancer is a major health issue all over the world. Cancer related deaths are one of the major causes of deaths, and are in > 90 % of the cases related to metastatic development, and spread of the cancer outside the primary location.

The receptor tyrosine kinase AXL is closely associated with the development of cancer and the receptor is upregulated in many different cancer forms. Upregulation is associated with increased invasiveness, and poor overall survival.

Warfarin is a known anticoagulant, which also is suitable as an AXL inhibitor. The warfarin-mediated inhibition of AXL is through the depletion of Vitamin K, with a subsequent inhibition of the γ -carboxylation of the Vitamin K dependent proteins in the body. GAS6, the ligand of AXL, is vitamin K dependent and will be unable to activate the receptor following warfarin treatment.

In this thesis, we have worked with the warfarin-mediated inhibition of the receptor tyrosine kinase AXL. In five different mouse model systems, we have evaluated the effect of warfarin-mediated AXL-inhibition in the development and metastasis of pancreatic ductal adenocarcinoma. We also evaluated how warfarin-mediated AXL inhibition impacts on expression of EMT markers, and the ability of the cells to migrate and form colonies, which is a hallmark of cancer with metastatic properties.

Further, we performed a register based cohort study using the Norwegian National Registry, the Cancer registry of Norway and the Norwegian prescription database. We investigated the cancer incidence in warfarin users compared to non-users in a broad segment of the Norwegian population, with a cohort comprising 1,2 million persons aged 52-82 years.

Our work establishes AXL as an important driver of metastatic formation in pancreatic ductal adenocarcinoma. The level of metastatic disease were significantly reduced in all warfarin treated animals. We also confirmed the close relation between AXL and EMT, as epithelial markers were upregulated when AXL was inhibited. The cells migratory and colony forming capabilities were also impaired after AXL inhibition.

In the population-based register study we observed an overall cancer protective association, with lowered incidence rate ratio of cancer in warfarin users compared to non-users. This was observed both for all-site cancer, and for the most prevalent cancer diagnoses in the material.

Altogether, our results emphasizes the importance of the receptor tyrosine kinase AXL in the development and progression of cancer. Warfarin-mediated AXL inhibition are shown to have a cancer protective effect, both in murine model systems and in population level studies. The results from this thesis suggest further investigations, to fully illuminate the potential use of warfarin in an anti-cancer setting.

List of publications

I

Kirane, A.*, Ludwig, K.*, Sorelle, N., **Haaland, G.**, Sandal, T., Ranaweera, R., Toombs, J., Wang, M., Dineen, Sean., Micklem, D., Dellinger, M., Lorens, J.B., Brekken. R.A.

Warfarin blocks GAS6-mediated AXL activation required for pancreatic cancer epithelial plasticity and metastasis. Cancer Res 2015; 75(18); 3699-705.

*Authors contributed equally to this work.

II

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Lower overall cancer incidence in patients treated with warfarin: A prospective population-based cohort study. (Manuscript submitted)

* Authors contributed equally to this work.

Other contributions not included in the thesis:

Kjersti T. Davidsen, **Gry S. Haaland**, Maria K. Lie, James B. Lorens, Agnete S.T. Engelsen

The role of AXL receptor tyrosine kinase in tumor cell plasticity and therapy resistance.
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1. Introduction

1.1 Cancer

The term cancer describes a diverse group of diseases. These diseases can present very differently, but share common features of uncontrolled cell division, and the ability of metastatic dissemination. The term malignant is used when cells in a tumor has the ability to invade either nearby, or distant tissues.¹ Cancer is a major health problem throughout the world. In Norway, there were 32,592 new cases of cancer reported in 2015. As shown in figure 1, the three most frequent cancer sites for men were prostate, lung and colon, and for women they were breast, colon and lung.

A MALES all ages (84 619 cases)

B FEMALE all ages (71 795 cases)

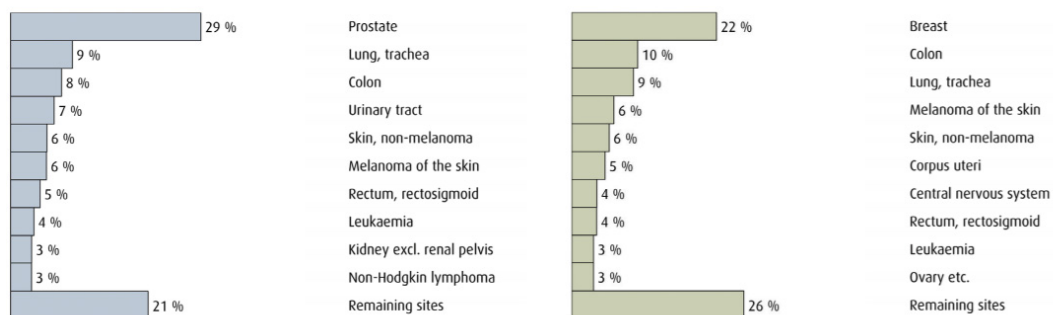


Figure 1: The most frequent cancer types in Norway 2011-2015. Adapted from²

In 2014, 10971 cancer deaths were reported, and death from cancer was the second most common cause of death after heart-diseases. For men, lung cancer (1198) and prostate cancer (1093) are the more frequent causes of cancer death, and lung (960), breast (663) and colon (595) are the most frequent in women.²

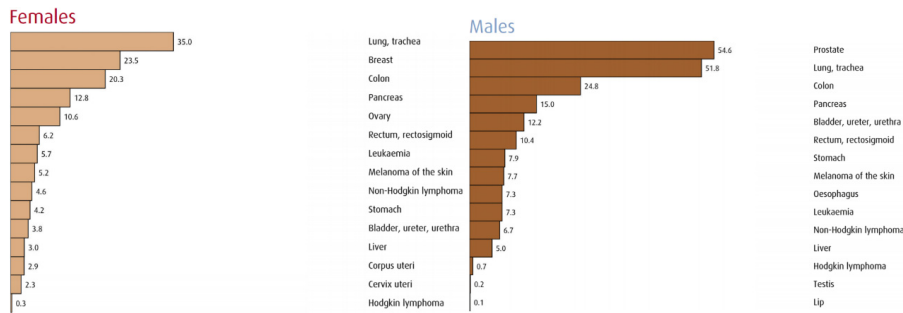


Figure 2: Age-standardized (Norwegian standard) mortality rates per 100 000 person-years for selected cancers in Norway, 2014. Adapted from²

1.2 Pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer death in Norway, with a 5-year relative survival of 6.4%.² Pancreatic cancer is the 12th most frequent cancer worldwide, but the high overall mortality, with 330,000 deaths in 2012 makes it to the seventh most leading cause of cancer death.³ There are several risk factors for developing pancreatic cancer, with increasing age as the major one. The cancer form is rarely seen before the age of 40 years, and the risk is 40 times increased at the age of 80 years. Family members of patients with pancreatic adenocarcinoma has an approximately threefold risk of developing the disease, suggesting a genetic inheritance.⁴ Also increasing body mass index, new onset diabetes mellitus, chronic pancreatitis, and smoking are factors known to increase the risk.⁵

The most common form for pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), with over 85% of all pancreatic neoplasms being of this origin.⁴ These tumors are located in the head of pancreas in 65% of the cases, and tumors with this localization is normally presenting earlier than other localizations, mainly with symptoms as acute pancreatitis, jaundice and/or biliary obstruction.⁵ Several mutations are linked to the progression of PDAC. The most described ones are activation of the oncogene *VI-KI-RAS2 Kirsten rat sarcoma viral oncogene homolog (KRAS)*, followed by inactivation of several tumor suppressor genes, such as *cyclin dependent kinase inhibitor 2A (CDKN2A)*, *SMAD family member no 4 (SMAD4)*/Deleted in pancreatic cancer -4 (DPC4),

Tumor protein p53 (TP53) and *Breast cancer 2 (BRCA2)*.^{4,6} In most cases, PDACs evolve through non-invasive precursor lesions, so-called pancreatic intraepithelial neoplasias (PanINs). These are microscopic lesions, (<5mm), and not detectable by non-invasive imaging. The PanINs are graded from 1 to 3 after level of cellular atypia. Low-grade PanINs (PanIN1) are increasingly common with increasing age. High-grade PanINs (PanIN3) are usually found together with invasive cancer.⁶ The genetic alterations associated with invasive cancer are also found in PanINs and the prevalence of these alterations will increase corresponding to the cytological and architectural atypia in the PanINs. *KRAS* gene mutations are normally one of the first alterations to be present in these lesions, and are increasingly frequent with the development of more advanced disease. At the stage of PDAC, nearly 100% of the tumors present with *KRAS* mutations.^{4,7}

1.2.1 Other pancreatic tumors

Neuroendocrine tumors

Pancreatic neuroendocrine tumors (PNET) are rare, representing 1-2% of all pancreatic tumors. They originate from pluripotent cells in the pancreatic ductal/acinar system. They could be secreting biologically active hormones, or they could be non-functional (60-90%). Hormone secreting tumors can give many different clinical syndromes, where hyperinsulinemia (insulin-secreting tumors), and Zollinger-Ellisons syndrome (gastrin-secreting tumors) are the most common forms.⁸ The malignant potential of these tumors vary from slow-growing tumors with non-invasive growth, to invasive and metastatic tumors.^{9,10}

Acinar cell carcinoma

Acinar cell carcinomas (ACCs) are rare neoplasms, accounting for 1-2% of all pancreatic tumors.¹¹ These tumors produce high amounts of digestive enzymes, which can give symptoms as skin rashes and joint pain.¹² The distinction between ACCs, and PNET can be unclear, and it is shown that one third of the ACCs have a neuroendocrine component.¹¹

Cystic neoplasms of pancreas

Malignant cystic tumors of pancreas represent 3-4% of pancreatic neoplasms and the most common forms are mucinous cystic neoplasms, serous cystic neoplasms and intra-ductal papillary mucinous neoplasms (IPMN). Mucinous cystic neoplasms are the most frequent type, representing 40% of the cystic neoplasms. The prognosis is similar to PDAC, except patients with IPMN can present with pre-invasive lesions, which have a more favorable prognosis.⁵

1.2.2 Staging of pancreatic cancer

Pancreatic cancer is staged from 0 to IV regarding size, borders and affection of surrounding tissue and/or lymph node and distant metastases.⁶ The staging is based on the Tumor, Node, Metastasis (TNM) classification of malignant tumors.¹³

Pancreatic cancer is staged at the point of diagnosis, and the staging will be determinative for choice of treatment.

| Stage | TNM | Category | Median survival |
|-------|---------------------------------------|----------------------------------|-----------------|
| 0 | Tis, N0, M0 | Local or resectable | 17-23 months |
| IA | T1, N0, M0 | Local or resectable | 17-23 months |
| IB | T2, N0, M0 | Local or resectable | 17-23 months |
| IIA | T3, N0, M0 | Local or resectable | 17-23 months |
| IIB | T1, N1, M0; T2, N1, M0; T3, N1, M0 | Local or resectable | 17-23 months |
| III | T4, any N, M0 | Locally advanced or unresectable | 8-14 months |
| IV | Any T, any N, M1 | Metastatic | 4-6 months |

Table 1: Staging of pancreatic cancer. Adapted from⁶

1.2.3 Treatment of pancreatic cancer

The treatment opportunities in advanced stages are few, and are yet not very effective.⁶ In the following section, the most common choices of treatment are described.

1.2.3.1 Surgery

Surgery, with complete surgical resection of the tumor is considered to be the only way to cure the disease. Unfortunately, surgery is possible in only ~10% of the patients.^{6,14} Metastases at the time of diagnosis is an absolute contraindication for operation.¹⁵ Even after radical surgery, the majority of the patients have a poor prognosis due to recurrence of tumor, and metastatic development.⁵

The methods used for surgical intervention, are pancreaticoduodenectomy (Whipples operation), distal resection of pancreas or total pancreatectomy. Operative mortality is low, and the procedure should be performed at centralized surgery wards, with preferably more than 15-20 pancreaticoduodenectomies per year. This is important to keep the complication rate as low as possible.⁶

1.2.3.2 Chemotherapy

Adjuvant treatment is given with the purpose to prevent, or delay, any recurrent disease. This treatment is given to patients after radical surgery with curative intention. The most used regimens are 5-fluorouracil and leucovorin, or gemcitabine, both for a period of 6 months. In some cases, neo-adjuvant chemotherapy is indicated, but this is still somewhat controversial, and is not yet recommended as standard treatment in Norway.¹⁶ Also FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin) could have a potential in an adjuvant setting, and this is currently in clinical trials (NCT01526135).¹⁷

In a palliative setting, FOLFIRINOX is first choice, when performance status is good. This gives an acceptable quality of life for most of the selected patients with relatively few side effects as long as the Eastern Cooperative Oncology Group (ECOG)-score is 0-1 before start. In addition, albumin-bound paclitaxel (nab-paclitaxel) and gemcitabine, or gemcitabine and capecitabine could be an option for patients when FOLFIRINOX is considered too toxic. In addition, gemcitabine monotherapy is an alternative for patients when therapy that is more intensive is not feasible due to co-morbidities or other complications. If first line treatment gives stable disease for a period of time, it is

possible to continue with second line treatment after progression. Gemcitabine or FLOX (fluorouracil and oxaliplatin) can both be used as second line treatment in these settings.^{14,18,19}

1.2.3.3 New drugs in development

Immunotherapy has shown encouraging results in early clinical trials, and different trials have different treatment approaches. Checkpoint inhibitors are promising, because of their ability to enhance the anti-tumor response of the immune system, and several clinical trials is currently ongoing in pancreatic cancer. Programmed Death protein 1 (PD1)/Programmed Death Ligand 1 (PD-L1) inhibitors or Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) inhibitors are given alone or in combination with each other or already established treatment (NCT02311361, NCT02868632, NCT02866383, NCT02777710, NCT02734160).²⁰ Therapeutic vaccines against pancreas cancer are also currently being tested, and furthermore, different monoclonal antibodies, cytokines and oncolytic virus therapies.²¹

1.3 Tumor Biology

Cancer development is a complex, multi-step process, normally developing over many years. The development of cancer requires changes, both regarding single cells and for their surroundings. Changes at cell level normally consists of mutations, deletions or up- or downregulation of regulatory proteins. An average tumor normally have 2-8 so-called driver gene mutations, providing the tumor with a growth advantage, in addition to 30-60 less important mutations.²² Also microenvironmental changes and changes in how the environment responds to an atypical cell, is required in the development of a fulminant cancer.²³ In 2000, Hanahan and Weinberg stated six hallmarks of cancer. (See figure 2)²⁴ In 2011, two new emerging hallmarks were proposed.(figure 2).²⁵

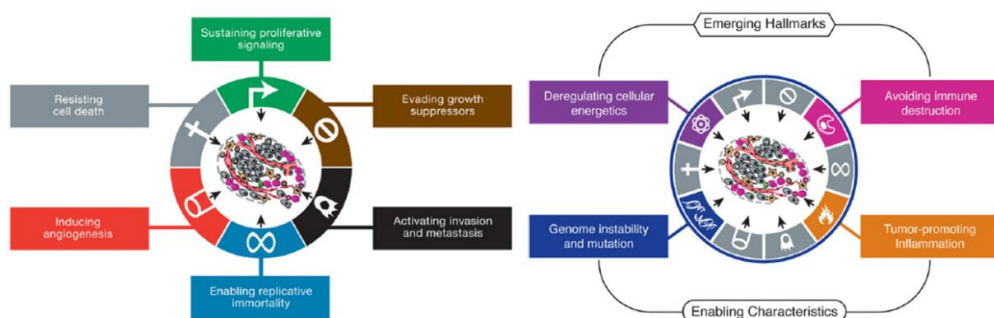


Figure 2: Hallmarks of cancer. Adapted from²⁵

Sustained proliferative signaling is one of the most important steps in cancer development, and there are many ways to achieve this. Common for many of the different strategies are their influence on the cell cycle. During a normal cell cycle, several checkpoints are established to control the properties of the cell before the entering of a new cell cycle state. In cancer, a common feature is that the cancer cell is continuously kept in an active proliferating state, without being withheld at the checkpoints and transferred to senescence.^{26,27} Cancer cells can produce self-made growth factors giving rise to an autocrine stimulation of proliferation. The cancer cells can also stimulate neighboring cells in the tumor stroma to produce growth factors, or overexpress receptor proteins on their surface to make them hyper-sensitive in situations when limited access to growth factors otherwise could stop the signaling.²⁸ In addition, ligand independent firing is possible due to structural changes in the receptors. Further, downstream pathways could be activated without receptor activation.²⁵ The mentioned cellular changes have the potential of making growth factors constitutively active, which will stimulate the cell to increased proliferation.²⁹

A cancer cell will also deactivate mechanisms in the cell designed to negatively regulate cell proliferation. One example is the tumor suppressor gene *TP53*, which under normal conditions controls the internal cell machinery, and stops further cell cycle progression if the cell is under stress, or has developed genomic damage. Stressful cell conditions can be hypoxia or suboptimal glucose access, and this can activate *TP53* and trigger

apoptosis.³⁰ Many cancers have different mutations in *TP53*, mainly missense substitutions, and this will alter the proteins ability to suppress cell growth.³¹ An inactivating mutation of *TP53* will lead to apoptosis evasion, and is seen in many human cancers.³¹

To be able to develop into macroscopic tumors, cancer cells will further need the ability to replicate unlimited. Telomeres are protecting the ends of chromosomes, but they are shortening in each division, until they no longer can protect the coding part of the DNA. Under normal circumstances, this will trigger a cell crisis, and subsequent cell death.²⁵ A majority of cancer cells expresses the enzyme telomerase reverse transcriptase (TERT), which will add segments to the telomeres located at the chromosome ends. This will prolong or even give the cells unlimited replication ability. Mutations in the promoter of the human *TERT* gene, is one of the most common noncoding cancer related mutations, although common cancers like breast and prostate will normally not have this mutation.³²

It is also essential for every tumor to have access to a sufficient amount of oxygen and nutrients. To achieve this, it is necessary with an adequate vasculature to supply the needs of the developing tumor. During cancer development, preexisting vasculature continues to develop new blood vessels, despite being quiescent under normal conditions. This is often referred to as an angiogenic switch.³³ Different tumors express different proangiogenic factors. Vascular endothelial Growth factor-A (VEGF-A) is the most widespread, but also fibroblast growth factor (FGF) and other members of the VEGF family are expressed in a cancer setting. Tumor blood vessels are different from normal vessels, being more irregular, dilated and with the occurrence of non-functional dead-ends.³⁴

The majority of the steps involved when a cell develops into a cancer cell, such as elevated levels of metabolic activity and cell division, is increasing the energy-needs of the cell. To meet the new requirements, the developing tumor cells are dependent of changes in energy metabolism. The Warburg effect, first described by Otto Heinrich

Warburg in 1956, is a metabolic switch observed in cancer cells. In this process, the normal Adenosine triphosphate (ATP)-production via oxidative phosphorylation changes to ATP-production via glycolysis, also under normal oxygen levels.^{35,36} Glycolytic ATP-generation is quicker, but is using more glucose than oxidative phosphorylation, which demands a high level of glucose supply from the surroundings. The increased glucose uptake in the tumor tissue is exploited for diagnostic purposes, with the imaging-technique of [¹⁸F] fluorodeoxyglucose positron emission tomography (FDG-PET).³⁷

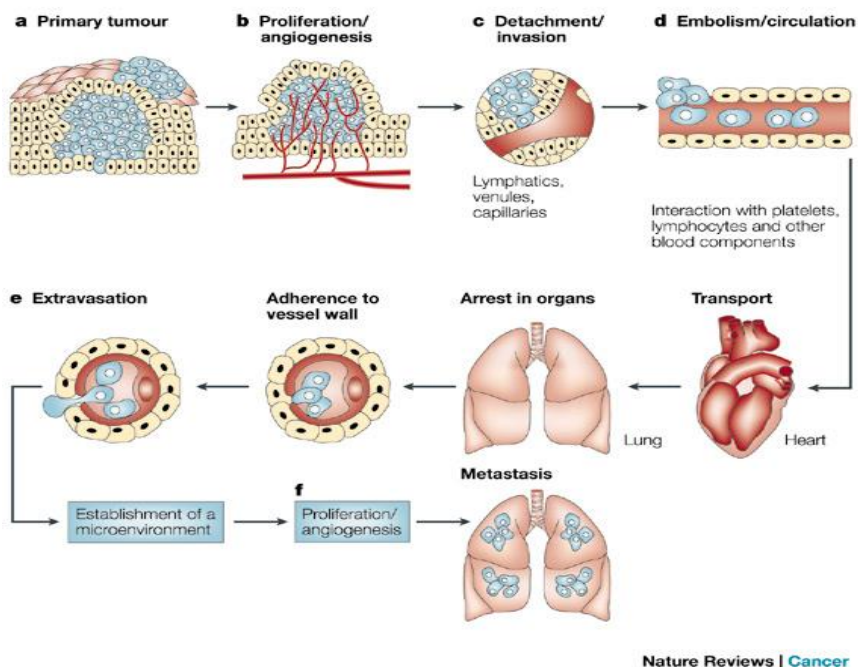
Altogether, a series of events is required in the development from a normal cell to a cancer cell. The process could stop in any of these steps, and this will stop the cancer from developing further. Different treatment approaches is also able to target different of these steps, aiming to stop the process. As an example, bevacizumab is a monoclonal antibody targeting VEGF, preventing the development of a sufficient tumor vasculature.³⁸ Also drugs that prevents cells for entering new cell cycles have been developed, targeting the key regulators of the cell cycle, the cyclin dependent kinases.³⁹

1.3.1 Mechanisms for metastases

Metastases account for >90 % of cancer related deaths.⁴⁰ An established metastatic tumor at a different and often distant site of the primary tumor is a result of a series of events, which involves local invasion, intravasation, circulative transportation, extravasation, micro-metastatic formation, and finally colonization and formation of a macroscopic metastasis.^{41,42} The metastatic process can stop in either of these steps, and the outcome is depending on properties of the tumor cell, but also on responses from the microenvironment at the new site.⁴² It is shown that only approximately 0.02 % of the cancer cells that enter circulation is developing into macroscopic metastases.⁴³ The potential of a cancer cells to metastasize is dependent on the degree of genomic instability in the cell. Cells with high grade of genomic instability will more easily acquire the alterations necessary to metastasize.⁴⁴ It is known that the epithelial mesenchymal

transition (EMT) is a driver of the metastatic process, and this will be discussed further in section 1.4.2.

Experiments have shown the metastatic potential of a tumor is closely related to increased resistance to apoptosis, which is considered as the initial step of the metastatic process.⁴⁰ The cell detaches from surrounding cells and extracellular matrix (ECM), and at the same time take a more rounded shape due to degradation of the actin skeleton. In normal conditions, these processes would lead to apoptotic cell (AC) death, through either anoikis (apoptosis induced by cell detaching) or amporphosis (apoptosis induced by disrupted intracellular architecture).⁴⁰ In a metastatic setting however, the abnormal cells will escape these processes, and continue to proliferate. It has been shown that overexpression of the anti-apoptotic protein B cell lymphoma 2 (BCL-2) increases the metastatic capacity of mammary epithelial cell, without affecting other important steps as primary tumor growth, cell motility or invasiveness.⁴⁵ Also the metastatic steps of intravasation, circulation, extravasation and establishment in a new micro-environment are promoted by anti-apoptotic mechanisms.⁴⁰ The steps of metastatic development are illustrated in Figure 3.



Nature Reviews | Cancer

Figure 3: The pathogenesis of cancer metastasis. The process of metastasis can stop in any of these steps. Adapted from⁴²

It is known that certain tumor types will metastasize to different specific organs. This problem was addressed as early as in 1889 with Paget's Seed and soil theory, where certain tumor cells (the seed) are hypothesized to have specific preferences for a certain micro environment in specific organs (the soil), independently of the rate of blood flow in the different organs.⁴⁶ A few years later, in the 1920s, another model of explanation were suggested. At this point, James Ewing proposed the theory of a circulatory pattern between primary tumor and metastatic organs. According to this theory, the metastatic sites are passive receptors of tumor cells, and the preferred organs depend on the circulatory network between the primary and metastatic site. Later experiments have confirmed that both theories could be valid, as the number and localization of metastases could depend both on mechanical factors as blood supply and tumor cell delivery, and also microenvironmental factor where the local environment at the metastatic site would favor growth of cancer cells from certain primary localizations.^{43,47}

To form a metastasis, the tumor cell has to survive in the new environment at the metastatic site. It is shown that AKT signaling is important in this matter, both when the tumor still is in circulation, but also in specific organs, as lung or bone marrow, to prevent the cell of undergoing apoptotic processes in the early phase of establishing a metastatic tumor.⁴⁸ Once localized at the new site, the metastatic cells have the capability of establishing a metastatic niche, in terms of releasing soluble factors or micro-vesicles to make the new microenvironment more facilitated for tumor development.⁴⁹ There is also hypothesized, although still debated, that tumor cells already at the primary site or in the circulation can secrete molecules to prepare the microenvironment at the site of metastasis, making a so-called pre-metastatic niche. This is in line with Paget's seed and soil theory.^{48,50} It is proposed that modifications of the stroma includes increased levels of fibronectin and matrix metalloproteinases (MMPs), structural changes in ECM and recruitments of bone marrow derived cells to make the environment more favorable for adhesion of the cancer cells, and subsequently metastatic colonization.^{51,52}

1.4 Epithelial to Mesenchymal transition

Epithelial to mesenchymal transition (EMT) is a process with cellular transformation from an epithelial to a mesenchymal phenotype. It is an important process in embryogenesis, which allows cells to migrate to different localizations during phases of development, both in morphogenesis and organogenesis.^{53,54} The embryonic form of EMT can be referred to as type 1 EMT. The EMT process is occurring also in adult tissue, both in normal processes such as wound healing and inflammation (Type 2), but also in pathological processes as such cancer, leading to cell invasion, dissemination, and development of therapeutic resistance (Type 3).⁵⁵ Characteristics of the EMT process are loss of cell polarity and cell-cell interactions, modulations of the adhesion between cells and ECM, enhanced proteolytic activity, ECM degradation, increased cell motility and reorganization of the cytoskeleton.^{56,57}

Epithelial cells have several features classifying them as epithelial. They have a well-defined apical-basal polarity with a basal membrane and widespread cell-cell contacts, such as tight junctions, which allows communication between the cells. They have a characteristic cobble stone-like shape, and are non-motile.^{58,59} Epithelial cadherin (E-cadherin) is an important protein responsible for the formation of adherence junctions, by making protein clusters connected to actin microfilaments. This provides a strong control of the epithelial architecture.⁶⁰ E-cadherin is considered the main marker for the epithelial phenotype, and in vitro, a correlation between the lack of E-cadherin and loss of an epithelial phenotype has been demonstrated.^{60,61} Other important cell-cell contacts are the tight junctions. The Claudin protein family is the most important component of the tight junctions, followed by the protein occludin. Both Claudin and Occludin are commonly used as markers for an epithelial phenotype, and they are shown to be downregulated during the process of EMT.^{53,57,62}

The phenotype of mesenchymal cells are quite different from epithelial cells. The shape is more elongated and spindle-like, and they do not have the strict apical-basal polarity seen in epithelial cells. They also lack the cell-cell contacts, which are critical in the epithelial cell structure. Furthermore, mesenchymal cells have the ability to migrate as single cells, and display another set of proteins than the epithelial cells, such as the mesenchymal markers Vimentin and N-cadherin.⁵⁹ In cancer, the levels of proteins that are characteristic of mesenchymal cells, and simultaneously loss of epithelial markers, correlates with evidence of tumor progression and poor prognosis.⁶³ Typically, the cells expressing mesenchymal markers are seen in the invasive front of primary tumors, and are most likely the cells that first will start disseminating.^{55,60,64}

1.4.1 Activation of EMT

The EMT program is activated by developmental transcriptional regulators. The most important of these are TWIST, Zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2), and two members of the snail superfamily of transcription factors, SNAIL (SNAI1) and SLUG (SNAI2).^{65,66} These transcription factors will change the gene expression

profile, by repression of the genes encoding for the epithelial proteins (e.g. E-cadherin and β -catenin) and induction of increased expression of mesenchymal proteins, such as Vimentin and N-cadherin. As an example, it is shown that SNAIL and ZEB bind to, and subsequently repress the activity of the *E-cadherin* promoter, and by that regulating the expression of E-cadherin.⁶⁷ SNAIL and ZEB are also contributing to destabilization of epithelial cellular polarity, which is a key feature of epithelial phenotype. By inducing expression of different metalloproteases that will degrade the basal membrane, they stimulate cellular instability and invasion.⁶⁸ Also expression of Claudins, which are important for tight junctions are downregulated by SNAIL. This is thought to be via the lysine specific demethylase 1 (LSD1).⁶⁹ There is also evidence for a positive feedback loop in this system. The metalloproteinase MMP3 will increase levels of reactive oxygen species (ROS) in the system, and that will again stimulate the expression of SNAIL.⁷⁰ Furthermore, it is shown that especially expression of SNAIL is closely related to signaling of transforming growth factor β (TGF- β).⁷¹ This is relevant, both during normal development, and in cancer.^{53,71} TGF- β has a two-sided role in the development of cancer. In many conditions, it is an important suppressor of epithelial cell proliferation and subsequently primary tumorigenesis. It will nevertheless serve as a positive regulator of tumor development in other conditions.⁷² During tumor progression, there is evidence that the tumor cells will lose their normal TGF- β -related growth inhibition, due to mutational changes. This will lead to increased growth, followed by more mutations, and subsequently cancer progression.⁷³ In a different pathway, there is evidence that the signaling protein Ras will be activated, and this will enhance the effects of TGF- β that promotes tumor progression, and metastatic development.⁷³ TGF- β also have the potential to activate the phosphatidylinositol 3OH kinase (PI3K) pathway with its downstream target AKT, which will lead to EMT –induction.⁷⁴ WNT signaling also have the potential of stimulating EMT. This large family of proteins are involved in several cancer types. Activation of the WNT pathway will phosphorylate Glycogen synthase kinase 3 beta, (GSK3 β), a tumor repressor, and this will via β -catenin activate transcription of *SNAIL*, and stimulate the EMT process.⁷⁴

1.4.2 EMT control

The process of EMT can be controlled by different factors. MicroRNAs (miR) are important in this respect. MiRs are small pieces of RNA (approximately 22 NT) which can bind to target mRNA and influence the translation. Especially, miR-200 is associated with EMT by regulating expression of ZEB.^{75,76} There is also evidence of down-regulation of miR-200 family members in several human cancers.⁷⁷⁻⁷⁹ Also SNAIL-dependent EMT can be regulated by miR, most commonly by the miR-34 family.^{80,81} Both the miR-200 family and the miR-34 family are controlled by the tumor suppressor TP53.^{81,82} TP53 will bind to the promoter of miRNA-200, and stimulate its expression. Loss of *TP53* in breast cancer will give less expression of miRNA-200, increased activation of the EMT-program and development of cells with stem-cells properties.⁸²

1.4.3 EMT and Cancer

In cancer development, EMT is thought to have an important role as a facilitator for dissemination and metastatic spread. This type of EMT is often referred to as type 3 EMT.⁵⁵ The process of EMT in cancer is strongly dependent of the tumor microenvironment, and micro-environmental factors will in many cases decide if a cell has the potential to undergo EMT and then metastasize.⁸³ The loss of E-cadherin during EMT is inversely correlated to cancer grade and patient survival, and E-cadherin downregulation is associated with increased cell growth.⁶⁰ A number of different growth factors will contribute to EMT in cancer, and the growth factor signaling will vary in different cell types. Examples of growth factors that can induce EMT are epidermal growth factors (EGFs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs) and as before mentioned, TGF- β .⁵⁴ Also activated Ras/mitogen activated protein kinases (MAPK), Src kinase and PI3K-signaling are shown to be inducers of the EMT program.⁵⁴ These pathways are associated with important hallmarks of cancer, such as ability to regulate cell cycle, sustained proliferation, and also the properties of evading growth suppression and apoptosis.²⁵ Other tumor-related factors can also trigger EMT in cancer. Intra-tumoral hypoxia could trigger the

expression of *SNAIL*, and subsequently the process of EMT. Thus, this is an important factor in tumor development, together with acidic conditions, inflammation and low blood glucose.^{54,84,62}

The role of EMT in cancer progression is still not fully understood. It is proven that EMT is not required for establishment of metastases, despite being very important for the invasive development.⁵⁷ Furthermore, cells with different EMT status could be present within the same tumor. It is hypothesized that there is an EMT gradient in different segments of the tumor, and that the cells goes through different states of intermediate EMT levels, where they gradually lose their epithelial phenotype.^{57,85} One theory is that a malignant tumor has an invasive front, where the cells have undergone EMT and have mesenchymal properties, whereas the main part of the tumor still largely is epithelial.⁵⁷

Both in embryogenesis, and in cancer development, EMT is a transient phenomenon. After cell dissemination and spread to distant sites, the cells will go through the opposite process, mesenchymal to epithelial transition (MET) in order to establish macro-metastases at the new localization.^{60,62} In metastases, the cancer cells exhibit histopathological similar traits as the cells in the primary tumor, lacking the mesenchymal phenotype, supporting the theory of the process of MET.⁵⁵ It is proposed that this is because the microenvironment in the new localizations will not provide the EMT stimulating signals present at the primary location, leading to a reversal of the process.⁸⁶

EMT is important also in other steps in the malignant development, such as resistance to cell death. TGF- β can induce EMT in mammary cells, and at the same time inhibit apoptosis. It is also shown that EMT induction can give rise to a phenotype with resistance of cell senescence induced by oncogenes.⁸⁷

1.4.3.1 EMT and Cancer stem cells

The EMT program has in several studies also been linked to the development of cancer stem cells (CSCs). A subset of the cells undergoing EMT is exhibiting stem-like properties, or being in a condition, just ready to enter the stem cell state.⁸⁸⁻⁹⁰ The hypothesis is that

these cells will exhibit some of the same traits as normal stem cells, such as the ability to self-renew and serve as progenitors for cell clones with adaptive characteristics.⁹¹ There is not consistency about the origin of cancer cells with stem-like traits, and different theories have been suggested. Dedifferentiation of the a cancer cell together with EMT is one of the proposed mechanisms, together with the hypothesis of malignant transformation of a normal stem cell, and induction of pluripotent cancer cells. In the invasive front of tumors there is evidence for expression of both stemness-associated genes, and EMT-related genes, supporting the theory of a dedifferentiation of a subpopulation of cancer cells together with EMT.⁹² It is shown that both the transcription factors SNAIL and TWIST stimulate the acquisition of stem cell properties in cancer cells.⁸⁷ Investigations of cells that have undergone EMT and exhibit mesenchymal markers show that a significant proportion of these cells will express cell surface markers compatible with stem cell traits, with a CD44high/CD24 low ratio.⁸⁸

1.4.3.2 EMT and drug resistance

It has been shown that the EMT-related transcription factor SNAIL can induce resistance to both chemotherapy and immunotherapy, and also immunosuppression. Furthermore, TWIST is capable of inducing resistance to senescence.^{87,93} Investigations of cell lines resistant to chemotherapy revealed that these cells present with a mesenchymal phenotype, and express markers of EMT.^{94,95} In acquired resistance, evidence shows that the process of EMT gives rise to more mesenchymal cells with chemo-refractory abilities and stem cell like features.⁹⁶ Also *de novo* resistance is related to EMT. In treatment of lung cancer with EGFR kinase inhibitors, sensitive tumors have elevated E-cadherin levels, while drug resistant cells have properties that are more mesenchymal.⁹⁷

1.5 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a group of transmembrane proteins, functioning as cell surface receptors and regulators of many cellular processes. In the human genome there are 58 RTKs, separated in 20 subfamilies.⁹⁸ The molecular structure of all

the RTKs are similar, containing an extracellular ligand-binding domain, and an intracellular tyrosine kinase region, separated by a transmembrane helix. RTKs are normally activated by ligand binding, followed by receptor dimerization and activation of an intracellular kinase domain.⁹⁸ Overexpression of RTKs, commonly because of gene amplification, is closely related to many human cancers.⁹⁹ Figure 4 gives an overview of human RTKs.

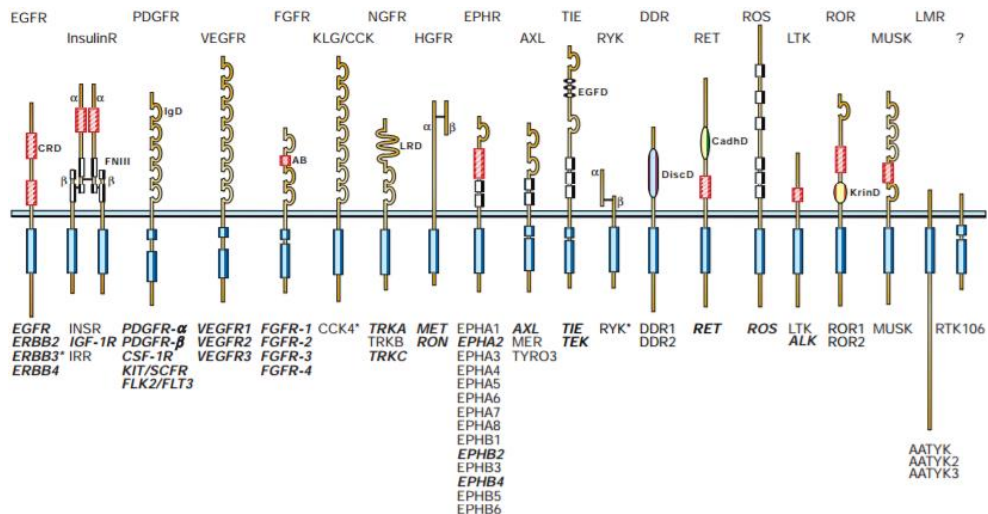


Figure 4: Human receptor tyrosine kinases. Adapted from⁹⁹

1.5.1 AXL receptor tyrosine kinase

AXL is a RTK and a member of the TAM (TYRO, AXL, MER), family of RTKs which is a group of transmembrane RTKs. AXL is located at chromosome 19, and is encoded by 20 exons. The receptor is approximately 140 kDa in a fully glycosylated state.^{100,101} The name AXL is from the Greek “anexelekto” which means uncontrolled.¹⁰⁰ AXL was first discovered in Chronic myelogenous leukemia, as an unidentified transforming gene.¹⁰² In normal tissues, AXL has a ubiquitous distribution. Detectable levels of AXL are found in endothelial cells, heart, kidney, liver, monocytes/macrophages, platelets, skeletal muscle, and testis. Also in the normal brain, there is evidence of AXL, most notably in cerebellum and hippocampus.¹⁰³

1.5.1.2 AXL structure

The structure of AXL is similar to other RTKs. AXL consists of an extracellular domain (N-terminal), containing two fibronectin type III domains, and two immunoglobulin (IG)-like domains.^{100,104} The intracellular tyrosine kinase domain (C-terminal) contains an unusual KWIAIE amino acid sequence, which is unique for the TAM family of RTKs. This sequence is similar, but different to the consensus sequence for all the tyrosine kinases.¹⁰⁰ The intracellular domain has an ATP-binding site, which catalyzes receptor auto-phosphorylation and serves as a docking site for cytoplasmic signaling proteins containing domains for protein tyrosine binding and src-homology-2.¹⁰⁵ This structure is common for all the TAM RTKs. Figure 5 shows the structure of AXL and its ligand GAS6.

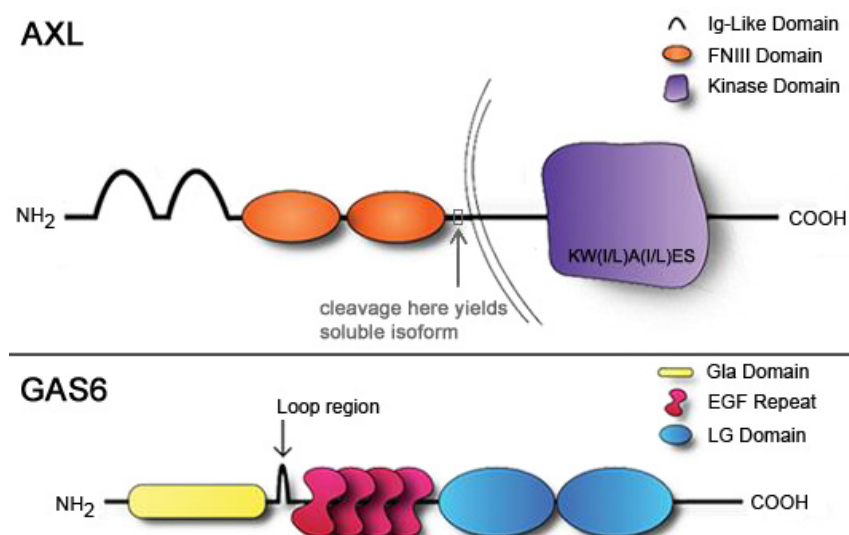


Figure 5: Structure of the receptor tyrosine kinase AXL and its ligand GAS6. Adapted from¹⁰⁶

1.5.1.3 AXL ligand

AXL is activated by its ligand Growth arrest specific 6 (GAS6) which was first identified in 1995.¹⁰⁷ GAS6 is a vitamin-K dependent protein, containing a gamma-glutamic acid residue (Gla) –rich domain at the N-terminal. The Gla-domain is under normal

conditions carboxylated from glutamate to gamma-carboxyglutamate prior to receptor binding. This vitamin-K dependent process is necessary for GAS6-mediated activation of AXL.¹⁰⁸⁻¹¹⁰ In addition to the Gla-domain, GAS6 also consists of four EGF-like repeats and a sex hormone binding globulin at the C-terminal, which includes two laminin G-like domains.¹¹¹ Structural studies have shown that there are two binding sites between AXL and GAS6, one major contact between LG1^{GAS6} and IG1^{AXL} and one minor contact between LG1^{GAS6} and IG2^{AXL}. Both the points of contact are required for AXL activation.¹¹²

1.5.1.4 AXL activation

After binding, AXL and GAS6 are creating a strong 1:1 GAS6:AXL complex, followed by a dimerization of two 1:1 GAS6:AXL complexes.¹¹² After gamma-carboxylation, the Gla-domain of GAS6 will bind to phosphatidylserine (PtdSer) on neighboring cells in a calcium-dependent process. Many cell types throughout the body are expressing PtdSer. In most conditions, PtdSer is located at the inner leaflet of the plasma membrane, but activated platelets and ACs are presenting the PtdSer on the outside.¹¹³ The interaction between the Gla-domain and the PtdSer on neighboring cells is optimizing receptor activation.¹¹⁰ There are conflicting reports regarding the possibility of AXL activation without simultaneously binding of PtdSer. The evidence is consistent in the findings of that the PtdSer binding is not essential for the binding of GAS6 to the receptor, but the presence of PtdSer is thought to increase the affinity.¹¹⁴ A study from 2014 argues that the gamma carboxylation of the Gla-domain is essential for receptor activation, but there is no need of following binding to PtdSer for receptor signaling.¹¹⁵ Another report from the same year argues that AXL signaling depends both on the carboxylation of GAS6 and subsequent binding of PtdSer.¹¹⁰ On the contrary, a more recent report demonstrates a possibility of receptor activation without gamma carboxylated Gla domain, and subsequently no binding to PtdSer but the activation will then be of weaker character.¹¹⁶ Figure 6 illustrates the GAS6-mediated activation of AXL.

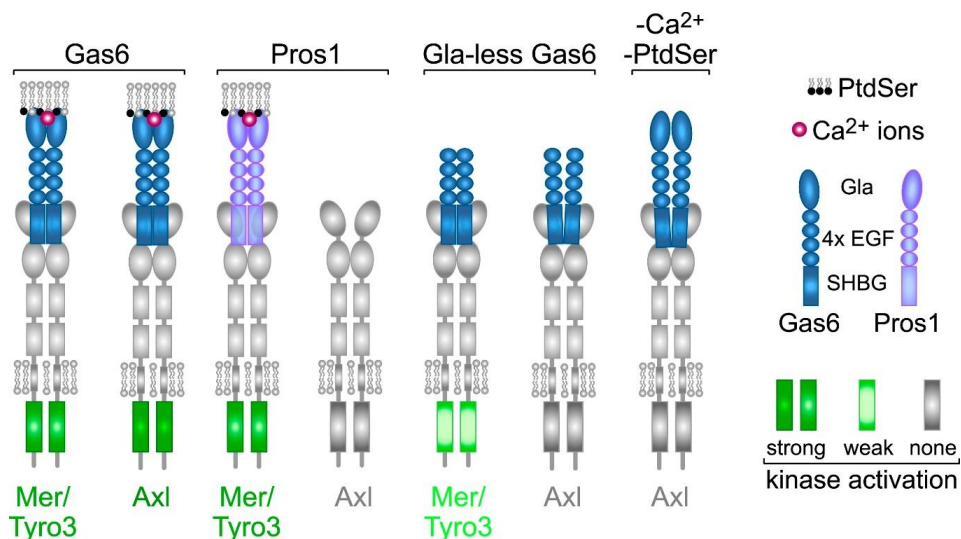


Figure 6: GAS6:AXL activation. Illustrates that AXL is activated of GAS6 and not Pros1, and that AXL is dependent of carboxylated Gla-domain of GAS6 binding to PtdSer on neighboring cells. Adapted from Lew et al¹¹⁰

There is also evidence that ligand-independent processes can activate AXL. This can be through interaction of two monomers on neighboring cells, causing cell aggregation, ligand independent dimerization and heterotypic receptor-dimerization with a non-TAM receptor.^{111,117} Ligand-independent activation of AXL is related to AXL overexpression, and is therefore more likely to occur under pathological conditions, such as cancer.^{118,119} Especially the homophilic binding of the extracellular domains on neighboring cells can lead to cell aggregation, and is associated with cancer, when the receptor is overexpressed.¹²⁰ Figure 7 illustrates the different mechanisms leading to AXL activation.

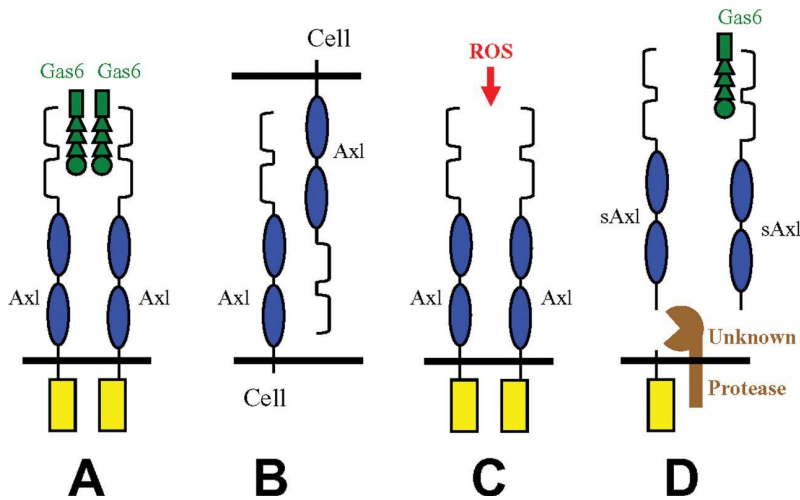


Figure 7: Mechanisms of AXL receptor activation/inactivation. **A:** Ligand induced activation. High affinity 1:1 GAS6:AXL complex followed by a dimerization of two GAS6:AXL complexes. **B:** Hemophilic binding of extracellular domain of AXL expressed on neighboring cells can lead to cell aggregation especially when AXL is overexpressed. **C:** Ligand independent hemophilic dimerization of AXL and auto-phosphorylation in response to ROS. **D:** Proteolytic cleavage of AXL to form soluble AXL (sAXL). Adapted from¹¹⁸

After the dimerization of the complexes, there will be a subsequent auto-phosphorylation of the intracellular kinase domain of AXL. There are several intracellular tyrosine residues which can be phosphorylated, the most described sites are Y779, Y821 and Y866, all located in the C-terminal kinase domain.^{118,121}

After activation of the GAS6:AXL complex, there is a cleavage of the extracellular domain from the cell surfaces, a process conducted by proteases.¹²² There is evidence that elevated soluble AXL (sAXL) in blood can be a marker of different conditions of variable character. For example, sAXL is suggested as a potential biomarker for inflammation, and early stage hepatocellular carcinoma.^{123,124}

1.5.1.5 Downstream events of AXL

The activation of AXL is linked to different intracellular signaling cascades, where several also are related to tumor development. The downstream signaling is thought to be similar to other RTKs, and different pathways are activated at different time points,

determined by tissue type, cell type and extracellular environment.¹⁰¹ The PI3K-pathway, with the downstream targets AKT and S6K, and also the phosphorylation of nuclear factor- κ B (NF- κ B) is one of the major downstream pathways of AXL activation. PI3K is an intracellular kinase and the key component of a pathway mediating several cellular responses related to cancer development, such as growth, motility and survival.¹²⁵ It is evidenced that the p85 (α and β) subunit of PI3K is interacting with a multi-substrate docking site at the tyrosine 821 on AXL.^{121,126} Activation of PI3K and subsequently NF- κ B, will lead to increased expression of anti-apoptotic proteins such as B-cell lymphoma 2 (BCL-2) and B-cell lymphoma extra-large (BCL-XL), and inhibition of pro-apoptotic proteins like caspase 3.¹⁰³ Furthermore, AKT will phosphorylate the pro-apoptotic BAD, which subsequently will not be able to interact with BCL-XL and BCL-2. This will lead to decreased apoptosis, and increased cell survival.¹²⁷ It has been shown in Gonadotropin releasing hormone -neurons that PI3K can mediate activation of p38, which in turn will lead to phosphorylation of Heat-shock protein 25. This is a regulator of actin modelling, and its activation will regulate remodeling of actin, which will favor increased migration.¹²⁸ Also p-21-activated kinases -1 (PAK1) is known to be activated through the PI3K/AKT pathway. PAK1 stimulates cell invasion when activated.¹²⁹ Another downstream target for AKT is GSK3. This protein has been shown to mediate several oncogenic traits, such as cell survival, proliferation and cell cycle progression.¹³⁰ Overall, AXL-mediated activation of the PI3K-pathway is linked to increased cell survival, proliferation and cell migration.

The MAPK/extracellular signal regulated kinases (ERK) signal transduction cascade is also initiated upon AXL phosphorylation. The tyrosine kinase domain of AXL will after auto-phosphorylation bind to the intracellular Grb2 protein, which then will activate the MAPK/ERK pathway.¹²¹ This pathway is often linked to AXL-mediated proliferation.^{103,131} This GAS6-mediated induction of ERK is both in strength and duration comparable to what is seen in response to more well-described growth factors, such as EGF and platelet derived growth factor.¹³² The growth-stimulating effect of GAS6 is additive to

the effect of EGF, which suggests that GAS6 utilizes other pathways than those utilized by EGF.^{126,132}

AXL signaling is heavily involved in the human immune system. Together with the other TAM receptors, the GAS6:AXL complex will protect innate immune cells such as macrophages, dendritic cells and natural killer cells (NK cells), from apoptosis. Via the type 1 interferon receptor (IFNAR), GAS6:AXL will initiate phosphorylation of the transcription factor STAT 1, a member of the Signal transducers and activators of transcription family of transcription factors, and subsequently the expression of suppressor of cytokine signaling 1 and 3 (SOCS 1 and 3), which are inhibitors of cytoplasmic cytokines. SOCS 1 and 3 will also inhibit Toll-like receptors (TLR) on dendritic cells, and by this inhibit their inflammatory response to pathogens. These pathways are very important for controlling inflammatory responses.^{118,133} Also STAT3 is linked to GAS6:AXL. STAT 3, as STAT1, belongs to the STAT family of transcription factors, and is persistently activated in many cancers. STAT 3 is known to be an important mediator of the oncogenic effects of EGF.¹³⁴ In head and neck-cancer and colorectal cancer, it is shown that inhibition of AXL will lead to reduced phosphorylation of STAT3.^{135,136}

Furthermore, there is shown a relationship between AXL and the cytokine interferon- α (IFN- α).¹⁰⁴ Secretion of IFN- α will upregulate AXL in macrophages, which in turn will lead to increased TWIST expression and reduced tumor necrosis factor α (TNF α) production. TNF α is a strong inflammatory cytokine, and reduced production will give a weaker inflammatory response.¹³⁷

The downstream events of AXL is illustrated in Figure 8.

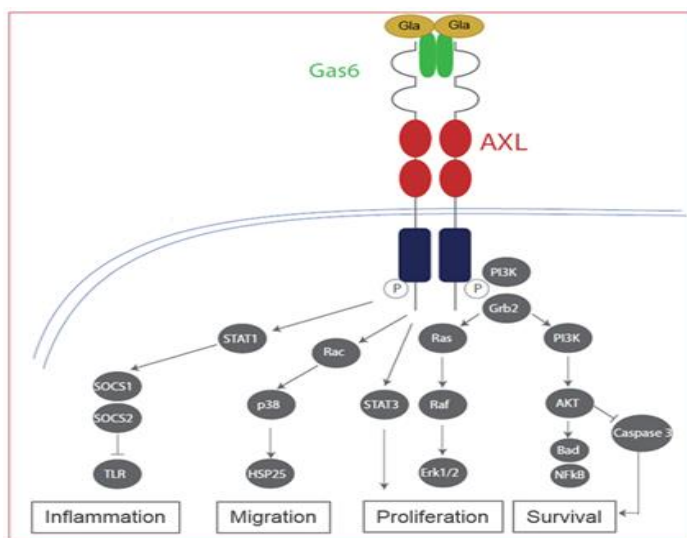


Figure 8: Downstream events of AXL. This figure show the downstream events after AXL activation. The boxes show the final outcome after the intracellular processes are activated.

1.5.1.6 AXL regulation

Some intracellular proteins have the ability to inhibit and regulate the activity of AXL. C1 domain containing phosphatase and TENsin homologue (C1-TEN) is a protein that will bind to the intracellular compartment of AXL. This will negatively regulate AXL signaling through PI3K/AKT. The detailed mechanisms behind this inhibition are still unclear. When overexpressed, C1-TEN will inhibit the cells ability to proliferate and migrate.¹³⁸ There is also evidenced that soluble forms of AXL (sAXL) are circulating in plasma, and will, by binding to GAS6, inhibit receptor activation.^{115,139} As other RTKs, AXL is regulated by a mono-ubiquitination signal, which leads to endosomal internalization and degradation by lysosomes. The regulating ligase for AXL internalization is Casitas B-lineage Lymphoma-c (c-Cbl).^{101,140} In kidney cancer it is shown that cells with low levels of VHL have increased AXL expression, due to binding of hypoxia-inducible transcription factor 1 and 2 (HIF-1 and HIF-2) to the AXL promoter.^{141,142} Myeloid Zinc finger 1 is another protein that is regulating AXL expression by binding to the promoter. This will activate the promoter, and increases the expression of AXL.¹⁴³

There is also evidence for AXL regulation at the mRNA level. Two miRs are identified (miR-34a and miR-199a) which binds to the 3'UTR of *AXL*, having an inhibitory effect on *AXL* protein levels.^{144,145} A recent study in ovarian cancer shows that expression of miR-34a will lead to decreased *AXL* expression followed by significantly inhibited cell migration.¹⁴⁶ Similar findings have been shown in prostate cancer where expression of miR-34a leads to downregulation of *AXL*, and induced apoptosis, and growth inhibition.¹⁴⁷ In osteosarcoma it is shown that miR-199a-3p will downregulate the expression of the *AXL* gene, and this will inhibit progression of the disease. Also, low levels of miR-199a-3p significantly correlates with recurrence of lung metastases, and low levels of miR-199a are a predictor of poor prognosis in osteosarcoma.¹⁴⁸ Specificity protein 1 and 3 (Sp1/Sp3) transcription factors are also regulators of *AXL* expression by binding to Sp motifs upstream for the *AXL* promoter, and by that driving *AXL* expression. In low *AXL*-expressing cells, these motifs are methylated which will restrict *AXL* transcription. This is in contrast to high *AXL*-expressing cells, where there is evidence for a hypo-methylation of the Sp motifs. Experiments with demethylation of these areas in low *AXL*-expressing cells, lead to increased *AXL* expression.¹⁴⁹

1.5.1.7 *AXL* in normal physiology

In the normal physiology, the expression of *AXL* is widespread throughout the body, although mainly in the mature immune, nervous, reproductive and vascular systems.¹⁵⁰ Still, none of the TAM receptors is essential for embryonic development, as TAM \neg/\neg are viable after birth.¹¹⁴

Hemostasis

AXL-expression on platelets will mediate thrombogenesis and platelet stabilization.¹⁵¹ The receptor will be activated by PtdSer on aggregating platelets, and simultaneous release of GAS6 from granules in the platelets. This process is contributing to stabilizing the clot formation. GAS6 \neg/\neg mice show signs of impaired platelet aggregation, with prolonged bleeding time.^{114,152} TAM RTKs are also involved in other parts of the vascular homeostasis, such as reestablishment of the endothelial barrier function after vascular damage, and also by promoting survival of endothelial cells.¹⁵³ Furthermore, *AXL* is

known to play a role in neovascularization, and it is shown that AXL is important for VEGF-A induced endothelial cell migration and subsequent formation of new blood vessels. This is effectuated through the downstream PI3K/AKT pathway.¹⁵⁴

Mediation of phagocytosis of apoptotic cells

In the process of phagocytosis, the TAM ligands GAS6 and Pros1 binds to PtdSer on ACs, and serves as a bridging molecule between the AC and a TAM-receptor on a neighboring phagocyte. After activation of the TAM-receptor, this linking of the cells will push the apoptotic process forward.¹⁰⁴ This is an important step in normal physiology, and necessary to prevent a state of continuous inflammation.¹⁵³ In AXL knockout (KO) animals the consequences is shown to be severe, with an accumulation of dead ACs. Especially regarding sperm production and in retina, this is important, leaving the AXL KO animals blind and sterile.¹¹³

Immunology

All the TAM receptors, have an important role in the inhibition of the innate immune system.¹¹⁴ The receptors functions as a safety-system to prevent prolonged and over-intense immune reactions and will promote tissue-repair after inflammatory responses. Dendritic cells have medium AXL expression at steady state, but the expression will be upregulated following pathogen invasion, which gives activation of TLR and further by type 1 IFN. This will contribute to the termination of the immune response after the specific pathogen reaction.¹³³ Situations with low AXL will always present with chronic inflammation and a prolonged immune reaction. AXL KO mice have severe autoimmune disease in a clinical pattern similar of systemic lupus erythematosus or rheumatoid arthritis.¹⁵⁵ When a virus enters the human organism, it is possible that AXL activation dampens the immune response, and thereby making it easier for the virus to escape the immune reaction. Many viruses have PtdSer on their external surface, and will by that activate AXL, and enter the cell. Infections like Zika virus, and also Ebola and West Nile virus has been coupled to AXL as a receptor for cell entrance.¹⁵⁶

1.5.1.8 AXL and EMT

AXL is known to be closely related to the EMT process. Over-expression of EMT-related transcription factors, such as *SLUG*, *SNAIL*, *TWIST* and *ZEB2* is linked to up-regulation of *AXL*.⁶⁵ *AXL* up-regulation will additionally have a positive feedback on the transcription factors, leading to sustained expression of *SLUG*, *SNAIL* and *TWIST*.^{65,157} There is also a tight connection between expression of EMT-related proteins and *AXL*. An example of this is the mesenchymal protein Vimentin. This is a protein is an important regulator of mesenchymal cell migration, and a marker of EMT. The level of *AXL* is closely related to the level of Vimentin, and increased expression of both proteins will enhance the cancer cells migratory capacity.¹⁵⁸ There is also evidence for a relationship between *AXL* up-regulation and acquisition of drug resistance and an EMT phenotype. This is described in several cancers, such as lung cancer, breast cancer and chronic myeloid leukemia (CML).¹⁵⁹⁻¹⁶² Furthermore, *AXL* expression is shown to be enhanced in breast cancer metastases relative to the primary tumor (investigated for matched samples). These findings strongly indicate that *AXL* is associated with epithelial plasticity and has a role in malignant progression and metastatic development.⁶⁵

1.5.1.9 AXL and cancer

AXL is associated with many different cancers. It was first described in Chronic myeloid leukemia (CML) in 1988. In the beginning, it was described as an unidentified transforming gene.^{102,163} Activating mutations or amplifications associated with *AXL* are rare, rather up-regulation and increased ligand-induced activation is associated with cancer.^{100,164} In the recent years, there have been many reports of *AXL* up-regulation in several different cancer types. (Table 1). It is believed that the upregulation of *AXL* is induced by hypoxia in the tumor environment, which is a common feature of most solid tumors. Hypoxic conditions will stimulate HIF-1 and HIF-2 to express several genes as response to this, amongst these, *AXL*.¹⁴² Overexpression of *AXL* has several implications. It is related to poorer prognosis^{65,165,166}, development of drug resistance^{159,161,167} and increased invasiveness.¹⁵⁸ Malignancies related to *AXL*-upregulation, and the correlation with poor prognosis are summarized in Table 2.

| Malignancies | Up-regulation | Human tumor | Poor prognosis | Independent prognostic factor |
|---------------------------------------|-------------------------|-----------------|-----------------|-------------------------------|
| Astrocytic brain tumors | 168-173 | 169,171,172 | 169 | 169 |
| Breast cancer | 65,174-185 | 65,176-183,186 | 65,182,183,185 | 65 |
| Gallbladder cancer | 187 | 187 | 187 | |
| GI cancers | | | | |
| ~Colon cancer | 136,143,144,188-191 | 136,190,191 | 136,191 | 191 |
| ~Esophageal cancer | 167,192-194 | 167,193,194 | 193,194 | |
| ~Gastric cancer | 195,196 | 195,196 | | |
| Gynecological cancers: | | | | |
| ~Ovarian cancer | 197-202 | 197-202 | 197,200,202 | 197 |
| ~Uterine cancer | 203-205 | 203-205 | 205 | |
| Head and neck cancer | 206-212 | 135,207,210-212 | 135,207,210,212 | 210,212 |
| Liver - HCC | 213-217 | 214,217 | 214,217 | 214,217 |
| Leukemias: | | | | |
| ~AML | 166,218-220 | 166,218-220 | 166,218 | 166,218 |
| ~CLL | 221-223 | 221-223 | | |
| ~CML | 100,162,220,224 | 100,220 | | |
| Lung cancer: | | | | |
| SCLC | 225 | | | |
| NSCLC | 144,159,165,188,226-228 | 159,165,231-234 | 230-232,234 | |
| Malignant melanoma | 235-238 | 238 | | |
| Mesothelioma | 239-241 | | | 239 |
| Pancreatic cancer | 109,242-244 | 242-244 | 242-244 | 242 |
| Sarcomas: | | | | |
| ~Ewing Sarcoma | | 245 | 245 | |
| ~Kaposi sarcoma | 246 | 246 | | |
| ~Liposarcoma | 247,248 | 247,248 | 247 | 247 |
| ~Osteosarcoma | 249-251 | 249 | 249 | 249 |
| ~Undifferentiated pleomorphic sarcoma | 252 | 252 | 252 | |
| Skin SCC | 253,254 | 253,254 | | |
| Thyroid cancer | 255-258 | 255,256,258 | | |
| Urological cancers: | | | | |
| ~Bladder cancer | 259-261 | | 259 | |
| ~Prostate cancer | 262-265 | 263,265 | | |
| ~RCC | 142,266-270 | 266-270 | 142,268 | 268 |

Abbreviations: HCC: Hepato-cellular carcinoma, AML: Acute myeloid leukemia, CLL: Chronic lymphatic leukemia, CML: Chronic myeloid leukemia, SCLC: Small cell lung carcinoma, NSCLC: Non small cell lung carcinoma, RCC: Renal cell carcinoma.

Table 2: AXL upregulation and correlation with prognosis. Adapted from ²⁷¹

Invasion and metastasis

Overexpression of AXL is thought to be more related to metastatic dissemination and poor overall survival than primary tumor growth.^{188,244} These observations correspond to the receptors close relation to EMT. AXL will enhance invasiveness in many cancers, which corresponds to the association to metastatic development. The AXL-related PI3K/AKT activation has in breast, gastric and ovarian cancer been shown to enhance tumor cell invasion, via the NF- κ B pathway.^{65,195,201} In hepatic carcinoma, AXL downregulation will lead to reduced expression and less activity in the PI3K/AKT pathway.²¹⁶ PAK1 is known to be activated through the PI3K/AKT pathway, and PAK1 stimulates cell invasion when activated.¹²⁹ Inhibition of the PI3K/AKT pathway and subsequently PAK1 were shown to strongly reduce cell invasion ability.²¹⁶ The E3 ubiquitin ligase Casitas B-lineage lymphoma-b (Cbl-b) is activating Natural Killer cells (NK-cells). By inhibiting this ligase, the NK-cells will be triggered to reject metastatic tumors. AXL, together with the rest of the TAM family, is shown to be substrate for Cbl-b, so by AXL inhibition, the activity of the ligase is inhibited, and the NK-cells will have an inhibitory effect of metastasis formation.²⁷²

Cell survival

Overexpression of AXL in tumor cells is in many conditions linked to increased cell survival. It is reported in different cancers that increased AXL expression leads to prevention of apoptosis, and subsequently increased survival. A work in breast cancer reports that elevated estrogen levels stimulate increased AXL expression and reduced levels of apoptosis.¹⁷⁶ Also in osteosarcoma there is shown a strong relationship between increased AXL expression and protection from apoptosis.²⁵¹ Similar reports is seen in astrocytoma¹⁷⁰, chronic lymphatic leukemia (CLL)²²² and ocular melanoma²³⁷. Some of the anti-apoptotic potential of AXL, goes through the PI3K pathway. Activation of PI3K and subsequently NF- κ B, will lead to increased expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL, and inhibition of pro-apoptotic proteins like caspase 3. In addition, the inhibitory interaction between BAD and Bcl-2 and Bcl-XL will be

blocked due to AKT-mediated phosphorylation of BAD. The total effect will be prevention of apoptosis and increased cell survival.^{103,127}

Angiogenesis

Furthermore, there is strong evidence supporting AXL having a role in angiogenesis.^{273,274} In the normal cellular environment, AXL is involved with repair of vascular injury.²⁷⁵ Studies have shown that overexpression of AXL in cancer is present not only in the tumor cells, but also in surrounding vascular cells.^{169,233} Vascular smooth muscle cells (VSMC) express GAS6, and exogenous application of GAS6 will stimulate proliferation and mobility of VSMC.²⁷⁶ There is also evidence that AXL knockdown in endothelial cells (HUVEC) will impair tube formation, and that AXL is an important driver of proliferation of HUVEC cells.²⁷³ Furthermore, it is shown that AXL will influence angiogenesis through modulation of signaling, via angiopoietin/Tie2w and Dickkopf related protein 3 (DKK3) pathways. These proteins are known regulators of angiogenesis. In addition, combination of AXL knockdown and anti-VEGF therapy resulted in enhanced inhibition of tube formation compared to anti-VEGF therapy alone.¹⁸⁸ Furthermore, Ruan et al show that AXL is essential for the VEGF-dependent activation of PI3K/AKT, supporting the evidence of AXL having important implications in angiogenesis.¹⁵⁴ In vivo mouse studies have further shown a strong relationship between AXL and angiogenesis, and suggest that AXL inhibition will suppress formation of new blood vessels.^{157,273}

Tumor microenvironment

AXL can also regulate factors in the tumor microenvironment. Malignant tumors have the ability of invading tumor-surrounding tissues. A crucial trait to achieve this is the capacity of producing matrix-degrading enzymes. MMPs are important enzymes regarding this. MMP-9 is a type IV collagenase, which degrades type IV collagen, an important structure in the basement membrane and ECM.²⁷⁷ It is shown that AXL enhances the expression of MMP-9, by regulating the promoter activity through the MAP kinase kinase MEK/ERK pathway.²⁷⁸ In the environment of a tumor, the conditions

are often hypoxic compared to the surrounding tissues. Hypoxia will increase the expression of HIF1 α which will then promote increased AXL transcription.¹⁴²

1.5.1.10 AXL and drug resistance

AXL is a facilitator for acquisition of drug resistance in many cancers. The mechanisms for this are not fully elucidated, but it is shown that AXL is significantly overexpressed in therapy resistant cancers compared to tumors in other stages.²⁷⁹ Especially in breast and lung cancer, this is thoroughly described.^{159,161,227,280} It is known that increased expression of RTKs is a compensatory reaction to therapy-induced inhibition of a specific signaling pathway. For AXL, this mechanism has been shown for both antimitotic drugs, and also targeted agents.²⁸¹ In lung cancer with a situation of AXL overexpression, AXL will dimerize with EGFR, and by that activate downstream effects leading to limited sensitivity for anti-EGFR therapy and acquired resistance for RTK inhibitors.²⁰⁶ In head and neck cancer there is evidence that AXL over-expression will give inhibition of the signaling of c-ABL/p73, and subsequently reduced response to DNA-damage and decreased levels of apoptosis, which in turn will give resistance against DNA-damaging drugs such as cisplatin.¹⁶⁷ Also in malignant melanoma, AXL is important in developing resistance to MAPK inhibitors in B-Raf proto-oncogene serine/threonine kinase (BRAF)-mutant melanomas, and targeted AXL-inhibition increases the effect of treatment with BRAF-inhibitors when given together.²⁸²

1.5.1.11 AXL and Immunotherapy

In normal physiology, it is known that AXL is important in dampening the immune system, preventing immune overreactions and auto-immunity.¹⁰⁴ In a cancer setting, with AXL overexpression, this is important, because AXL would facilitate for the tumor cell to survive attacks from the immune system.²⁸³ AXL-inhibition will activate the immune system in several manners. For example, NK-cells will be stimulated to anti-metastatic activity and make it more likely for the tumor cells to be killed in an immune reaction.²⁷² There will also be an increase in activation of dendritic cells, an increase in proinflammatory and anti-tumoral cytokines, and promotion of intra-tumoral infiltration of cytotoxic cells.²⁸⁴ All these events will make it more likely for a tumor cell

to be rejected by the immune system. *AXL* overexpression is related to development of resistance to PD1-inhibition, and the hypothesis is that *AXL* inhibition will enhance the effect of checkpoint inhibitors.²⁸⁵ This is currently in clinical trial (NCT02872259). The potential of *AXL* inhibition in a cancer immunotherapy setting is addressed in a recent review, and more research to illuminate this further is of great importance.²⁸⁴

1.5.1.12 *AXL* and cancer stem cells

There is also evidence for a correlation between *AXL* and CSCs. A study regarding breast cancer stem cells (BCSCs) showed that *AXL* was capable of inducing EMT, by upregulating expression of Vimentin and N-cadherin and downregulation of E-cadherin in BCSCs. *AXL* also regulates the tumorigenicity and ability of invasion and migration of BCSCs.²⁸⁶ Cells with high *AXL* expression also have high surface-expression of CD44, which is a known marker of stem-cells. CD44 low cells, have less *AXL* expression, arguing that *AXL* expression is linked to the development of cancer stem cell traits.²⁸⁷

1.6 Vitamin K

Vitamin K was discovered in the 1930s, first described by Henrik Dam, a Danish scientist, who received the Nobel prize for the discovery. The major role of the vitamin is to serve as a co-factor in γ -carboxylation of glutamic acid residues in specific vitamin K dependent proteins (VKDP). The most known VKDPs are related to blood coagulation, but they are also found in bone homeostasis and the vasculature, preventing calcification.²⁸⁸ The known VKDPs are coagulation factors II, VII, IX and X, protein S, protein Z and protein C, and further GAS6, osteocalcin, matrix Gla protein and Gla rich protein, together with four unknown integral proteins (PRGP1-4).²⁸⁹ The Gla domain on VKDPs gives the proteins the ability to bind metal-ions. After binding of calcium-ions, the protein will change structural conformation in the matter of gaining the ability to bind to phospholipids. This is necessary for membrane binding, which is a known feature of these proteins.²⁹⁰ Dietary supplements of vitamin K is essential. Vitamin K is fat-soluble, and has several subtypes, where vitamin K₁ (phylloquinone) is the most available in the human diet, through plant-based nutrients, such as green vegetables, grains, fruits and dairy products.²⁹¹ Vitamin K₂ (menaquinone) is synthesized by bacteria, and can also be available for humans, either from gut flora bacteria, or from bacteria within food, for example cheese, or from liver-products.^{291,292} Menaquinone is a group of proteins, where 7 of the subgroups is considered relevant for human intake (menaquinones 4-10). Interestingly, menaquinon-4 can also be produced in mammals, with a conversion of vitamin K₁, or other menaquinones.²⁹³ This reaction is catalyzed by an enzyme called UBIAD1, which was first described in 2010.²⁹⁴ It is suggested that vitamin K₁ mainly is taken up in the liver, and vitamin K₂ in arteries and other extra-hepatic locations.²⁸⁹ As vitamin K is taken up in the body in the quinone form, it is necessary to convert it to the reduced form, hydroquinone, or vitamin KH₂ to make it biologically active. This step is catalyzed by the enzyme vitamin K epoxide reductase (VKOR), which was first discovered in 2004.²⁹⁵ Alternatively, the reaction

could be catalyzed by a more specific NAD(P)H-dependent quinone reductase. This enzyme is mainly active in the liver.²⁹¹ The Vitamin K cycle is illustrated in Figure 9.

The enzyme mediating the γ -carboxylation of glutamic residues is called γ -glutamyl carboxylase (GGCX). In the carboxylation reaction, one molecule of Glutamate (Glu) on the VKDPs is converted to γ -carboxyglutamate (Gla), at the same time as one molecule of vitamin KH_2 is converted to its inactive form, vitamin K epoxide. (See figure 9). In the conversion of Glu to Gla, one molecule of CO_2 is incorporated into the glutamic acid binding to γ -carbon.²⁹⁰ To be suitable for reuse as carboxylation substrate, vitamin K epoxide has to be converted back to its reduced form, a reaction catalyzed by VKOR.²⁸⁹ This recycling of vitamin K is preventing clinical vitamin K deficiency in humans, which is a rare condition.²⁹⁶

The liver is the site of synthesis of coagulation factors, and a substantial part of the vitamin K in the body are stored here. 10 % of the stored vitamin K is vitamin K_1 , the rest is vitamin K_2 , mainly MK-7-MK-13.²⁹² The heart and pancreas also contains comparable levels of vitamin K_1 as in the liver, and lower levels are observed in the brain, kidneys and lungs. Vitamin K_2 are found at significant levels in the brain, kidney and pancreas.²⁹¹

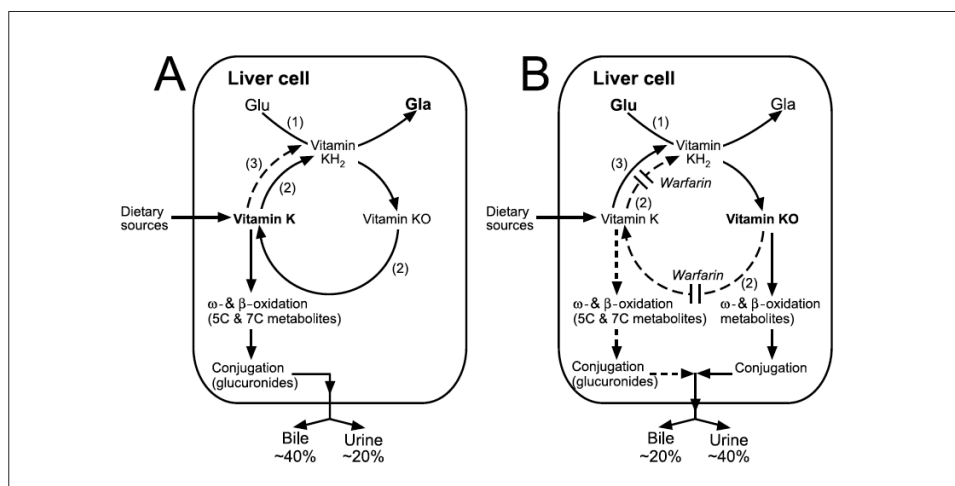


Figure 9: Schematic presentation of hepatic vitamin K metabolism. A: In normal conditions. B: In presence of warfarin. 1) γ -glutamyl carboxylase, 2) VKOR. 3) NAD(P)H-dependent quinone reductase. Figure adapted from ²⁹¹

1.7 Warfarin

Warfarin is one of the most used oral anticoagulants worldwide. Statistics from the Norwegian Prescription Database shows that it was approximately 70,000 daily users in Norway in 2015.²⁹⁷ Several studies show that the drug is used by approx. 2-10% of the adult population, with increasing prevalence with increasing age.^{298,299}

The clinical available form of warfarin is a racemic mix of equal amounts of (R)- and (S)-enantiomers, where the S-enantiomers is 3 to 5 times more potent.³⁰⁰ It is almost completely absorbed after oral administration and 98-99% is bound to plasma protein, especially albumin.³⁰¹ Warfarin reaches peak plasma concentration after 4 hours.³⁰² It is almost completely metabolized in the liver, mostly by CYP2C9.^{300,301}

Warfarin acts as a vitamin-K antagonist. It interferes with the cyclic conversion of vitamin K epoxide to vitamin K. This process is catalyzed by the enzyme vitamin K epoxide reductase (VKOR), which is inhibited by warfarin. Consequently, this will stop the regeneration of vitamin K from its inactive form, and lead to a depletion of active Vitamin K in the body.^{301,303} Warfarin shares a common ring structure with vitamin K_{H2}, and can by binding to VKOR inhibit the reductase reaction.²⁹⁰ There has been a discussion if there is a competitive or non-competitive binding of warfarin to VKOR, but recent research is suggesting a shared binding site for warfarin and vitamin K, and consequently a competitive binding to the enzyme.³⁰⁴

1.7.1 Cancer protective effects of warfarin in a historical perspective

Reports about anti-cancer effects of warfarin have been published occasionally the last 50 years. In 1968, Ryan et al reported a reduced incidence of spontaneous metastases after Coumadin (warfarin) therapy.³⁰⁵ The group performed a subcutaneous mouse model with anaplastic sarcoma and mammary adenocarcinoma, and evaluated the development of pulmonary metastases. In both models, there was a significant reduction in the metastatic formation in the Coumadin (warfarin) treated group. In the early years, the prevailing hypothesis was that the observed cancer protective effect of

warfarin was due to anticoagulation effects. This was also supported by observed cancer protective effects of other anticoagulants, such as unfractionated, and later, low molecular weight heparins.^{306,307} Brown et al further strengthened the hypothesis of a cancer protective effect of warfarin in a publication from 1973. The group showed that warfarin effectively reduced the number of lung metastases in a model of KHT sarcoma in mice, and that this effect was abolished when Vitamin K was administered together with warfarin. The authors attributes the effect to the coagulation system, and not as a direct effect of warfarin on the tumor cells.³⁰⁸ Also McCulloch et al concluded that the observed cancer protective effect of warfarin was related to anticoagulation.³⁰⁹ There was little knowledge about the molecular effect behind the anti-coagulative properties of warfarin during these first years. The connection with warfarin and Vitamin K was not properly established until a publication from O'Reilly et al in 1976, where they acknowledge the inhibitory effect of warfarin on the conversion of Vitamin K epoxide, to Vitamin K.³¹⁰ In parallel, theories about a direct warfarin effect on the tumor cells gradually started to emerge. In 1985, Goeting et al. observed an effect on colorectal cancer incidence in rats, both when warfarin was administered in doses affecting the coagulation system, but also in a lower, subclinical dose. The authors suggested that the observed effect was not related to the coagulation system, rather a molecular effect on early stage neoplastic changes.³¹¹ The coupling between warfarin and AXL inhibition was made in 1999, first from warfarin mediated AXL inhibition of mesangial cell proliferation in the kidneys.³¹² This connection was further established when Tsou et al showed that γ -carboxylation was necessary for AXL activation and that this process could be inhibited by warfarin¹¹⁵

1.7.2 AXL and warfarin

As described before, the RTK AXL, is dependent on the vitamin K dependent carboxylation of its ligand GAS6 for biological activation. By its inhibition of VKOR, warfarin will give a depletion of vitamin K followed by prevented carboxylation of GAS6 and subsequent inhibited receptor activation. Non-carboxylated GAS6 will have full

capability of binding to the receptor, but will function as a selective AXL-antagonist, as it in this state will not be able to induce receptor activation.³¹³ Warfarin has also been shown to enhance activity of NK-cells, at doses not affecting the coagulation system.²⁷²

1.8 Health registries

A health register is defined as a “collection of health information, systematically stored and organized so it will be possible to retrieve health information about individuals.”³¹⁴ In total there are 15 centrally administered health registries in Norway, covering topics as birth, drug prescriptions, cancer occurrences and causes of death.³¹⁵ The registries are regulated by the Norwegian law on Health registries, “Lov om helseregistre og behandling av helseopplysninger”.³¹⁶ The free, universal health care system in Norway gives the Norwegian health registries a broad, almost complete, coverage. All inhabitants of Norway are assigned their own unique identification number, registered in the Norwegian National registry, a system in use since 1968.³¹⁷ The use of this number is allowing an individual-level linkage of information from different health registries. This gives a wide variety of opportunities to conduct experiments and extract information from these sources.

1.8.1 The cancer registry of Norway

The Cancer registry of Norway (CRN) was established in 1951 and is after the cause of death registry the oldest health registry in Norway. The registry is collecting information on all cancer cases in Norway, and all medical doctors are instructed by law to notify new cancer cases to the registry. Also cases with cancer suspicion without verified diagnosis, and diagnoses revealed at autopsy should be reported to the registry.³¹⁸ The overall coverage is estimated to be over 98%. It records detailed information including demographic information, diagnosis, death by cancer, morphology, stage and topography. The registry uses International classification of diseases (ICD), ICD-7, and ICD-10 for diagnosis classification and the international classification of diseases for

oncology, 3rd edition (ICD-O-3), for morphological classification of the different lesions.³¹⁹

1.8.2 The Norwegian prescription database

The Norwegian prescription database (NorPD) was established in 2004, and collects data from all prescribed drugs in Norwegian pharmacies.³²⁰ The registry does not include information on drugs used by hospitalized or otherwise institutionalized patients, nor over the counter drugs, bought without prescription. The registry is using the Anatomical Therapeutic Chemical classification system for classification of the different drugs.³²⁰ When using NorPD for research, the database has several strengths compared to other sources of drug use, with no recall bias, and no primary non-compliance. This will improve the validity of the data of interest.³²¹

2. Aims of the study

The central hypothesis of this thesis is that AXL mediated signaling is important for the development and progression of cancer, and that warfarin-mediated AXL inhibition effectively blocks tumor initiation and malignant progression in different cancers.

Main aim: Characterize the role of warfarin-mediated AXL-inhibition in the development and progression of cancer.

Specific aims:

Aim I: Conduct AXL inhibition in pancreatic cancer through warfarin mediated inhibition of GAS6-carboxylation in different mouse models. (**Paper I**)

Aim II: Characterize AXL expression and expression of EMT markers in in vitro pancreatic models, with and without AXL inhibition, to investigate the relationship between AXL expression and EMT. (**Paper I**)

Aim III: Characterize cancer incidence in warfarin users compared to non-users to illuminate the potential effect of warfarin-based AXL inhibition in cancer development. Conduct a prospective population based cohort study, using the Norwegian Cancer registry and the Norwegian prescription database. (**Paper II**)

3. Summary of papers

Paper I

In this paper, we report that the vitamin-K antagonist warfarin blocks GAS6-mediated activation of the receptor tyrosine kinase AXL in different models of pancreatic cancer. This inhibition reduces both progression and spread of pancreatic cancer. In vivo experiments were performed with human cancer cells and immunocompromised mice, murine cells and immunocompetent mice, and with genetically engineered mouse models (GEMMs), that spontaneously developed PDAC. In all models, we saw a major reduction in the formation of metastases. We also demonstrated an increased effect of gemcitabine when given in combination with warfarin, both in primary tumor growth and metastatic development. The effect of warfarin mediated AXL-inhibition was verified also with other AXL-blocking agents. Through in vitro-experiments, we demonstrated that levels of phosphorylated AXL went down after treatment of warfarin. In addition, phosphorylation of AKT, downstream of AXL, was increased when stimulated with GAS6, and reduced after addition of warfarin. This confirmed that warfarin treatment reduced AXL signaling. Further, the cells ability to form colonies were significantly reduced after warfarin treatment, and these results were confirmed by AXL-knock down cell lines. We also established the close link between AXL and EMT in line with previously published material. Levels of EMT markers were influenced after warfarin-mediated AXL-inhibition, with vimentin being downregulated and E-cadherin being upregulated in warfarin-treated samples.

Paper II

We report in this paper a clear association between warfarin use and cancer. We defined a cohort with patients from the Norwegian national registry coupled with the Cancer Registry of Norway and the Norwegian Prescription Database to look at the incidence of cancer in warfarin users compared to non-users. We observed a

significantly lower cancer incidence in the warfarin user group, with an incidence rate ratio (IRR) for overall cancer of 0.84 (95% Confidence interval (CI) 0.82-0.87) adjusted for sex and age. We further observed a lowered IRR in several of the organ-specific cancer sites, such as prostate, lung and bladder cancer. We also performed a subgroup analysis on patients prescribed warfarin for atrial fibrillation/flutter (AF-group), to eliminate the possible confounding effect of occult malignancy after venous thromboembolism and pulmonary embolism. In these analyses the IRR in overall cancer were even lowered with an IRR of 0.62 (95% CI 0.59-0.65). Also for specific cancer sites, IRR were lowered in the user group compared to the non-users. The findings in this study supports the findings from **Paper I**, and emphasize the potential of warfarin use in an anti-cancer setting.

4. Methodological considerations

4.1 Animal experiments

The animal experiments in **Paper I** has been performed with different approaches. In the experiments, we have used immunocompetent animals, immunocompromised animals and also genetically engineered mouse models (GEMMs)

4.1.1 Cell line xenograft models

These models depend on the use of human cancer cell lines in mouse models, which is a common way of modeling cancer development.³²² A prerequisite for this model is the use of immunodeficient mice, to prevent rejection of the injected human cells. This way of modeling cancer has several challenges. The use of immortalized cell lines will not reflect the diversity in a normal tumor, as these cells are preselected cells grown in a favorable environment and often with a different gene expression profile than primary tumor cells.³²³ Furthermore, the immune system is important in normal cancer development, and in these models, this factor is eliminated. This makes it impossible to investigate immune-targeted therapies in this setting. Advantages of this model system are the access to numerous and well established cell lines, in many different tumor types. These cells can be injected both subcutaneously and orthotopically.

4.1.2 Syngeneic models

In this model, murine cancer cells are transplanted into mice, using a immunocompetent host. With an intact immune system, this will mimic a more realistic tumor environment, including stromal cells and tumor vasculature. Disadvantages of the model is that it is less available, as fewer cell lines suitable for the purpose exists.

The clinical translation, with both the tumor and the host being of another species than human is another challenge.³²⁴

4.1.3 Genetically engineered mouse models

In these cancer models, the mice are genetically altered, so that they spontaneously will develop the tumor of interest. This has several advantages with the tumor developing in the tissue of origin, and preservation of an intact immune system. The tumor microenvironment will also be intact, with all components such as immune cells, vascular and stromal cells.³²⁵ The major disadvantage of this model system is the complexity of developing the mouse model.³²⁴

4.2 Mouse strains in use in our work

NOD/SCID: Non-obese diabetics/ Severe combined immunodeficient mice. This mouse strain is immunocompromised, due to impaired development of T and B cell lymphocytes. In addition, these animals have reduced NK-cells function.³²⁶ These mice are widely used, both for tumor biology and xenograft research.³²⁷ In our work, the strain was used for orthotopic implantation of human cancer cell lines.

C57/BL6: This is an immunocompetent inbred mouse-strain. The strain was first bred in the Jackson laboratory in 1948. With a normal immune system, these mice are widely used in research in the fields of both immunology and cancer. They are robust and long-lived compared to other cancer models.³²⁸ We used this mouse model, in order to implant murine cancer cells, which gave us the ability to study the progression of PDAC in a model with a functioning immune system.

LSL-*Kras*^{G12D}; *Cdkn2a*^{lox/lox}; *p48*^{Cre} (KIC): Genetically engineered mouse strain. This model takes advantage of the pancreas selective transcription factor p48 (Ptf1a). This transcription factor drives the expression of Cre in pancreatic cells. The LSL-*Kras*^{G12D}

gives a mutant *Kras*, which due to the p48 Cre mutation is specifically expressed in the pancreas, promoting the development of PDAC. Furthermore, the *p16/p19* (*Ink4a/Cdkn2a*) locus is deleted, a common mutational loss in human PDAC. This gives the tumor an aggressive phenotype, with poorly differentiated tumor cells.³²⁹ The development of pancreatic carcinoma in this model is 100% at 4 weeks of age. The model has histopathological features that is consistent with the development and progression of human PDAC, and therefore works well as a model system.^{330,331} The mice were bred in the animal facility of UT Southwestern, Dallas, and genotyped shortly after birth.

We used the following pancreatic cell lines for our experiments:

Human cell lines:

AsPc-1: This cell line is derived from cells from the ascites of a patient with pancreatic cancer.³³²

Panc-1: This cell line was derived from a primary tumor. The cells do not express significant amounts of carcinoembryonic antigen.³³²

C5LM2: A variant of Panc1 cells. The cell line was developed by two passages of in vivo growth and culture of liver metastases from a primary pancreatic cancer. The cell line was developed in the Brekken laboratory.

Mia PaCa2: Cells from primary tumor of pancreatic cancer. This tumor did not express carcinoembryonic antigen.³³²

Capan1: Derived from a PDAC liver metastasis.³³² Capan 1 does not express AXL. Included as a control cell line.

Murine cell line:

Pan02: Murine cell line, established from pancreatic tumor in a C57/Bl6 strain. Widely used for research on pancreatic cancer.³³³

All cell lines for animal experiments were grown in a humidified atmosphere with 5% CO₂, at 37°C. AsPC-1, Panc-1, Pan02 and MiaPaCa-2 lines were grown in Dulbecco's modified eagle medium (DMEM). Capan-1 cells were grown in Iscove's modified Dulbecco's medium (IMDM). Before implantation, all cell lines were confirmed to be mycoplasma free using e-Myco kit (Boca scientific).

4.3 In vivo experiments

Animal experiments were performed at University of Texas Southwestern, Dallas, Texas. All animals were housed in a pathogen free facility. The animals had 24-hour access to food and water.

All cells were injected orthotopically. For AsPc-1, Panc-1, Mia PaCa2, Capan1 1×10^6 cells were injected, and for Pan02 cells 1×10^5 cells were injected.

4.3.1 Medical treatment of animals

The animals were randomized to receive normal drinking water, or water containing warfarin. For immunocompromised mice the warfarin concentration was 1 mg/L (3,0 μ M). Immunocompetent mice received 0,5 mg/L (1,5 μ M). Warfarin containing water was in all cases renewed every 3 days. The warfarin treatment were administered with or without gemcitabine 25 mg/kg twice weekly. For Mia Paca2 tumor bearing mice, also 10C9 (250 μ g ip. twice/week) were given in addition to gemcitabine.

The GEMMs started warfarin treatment at three weeks of age. The warfarin treatment continued for 4 weeks until sacrifice.

Mice implanted with Panc-1, Capan-1, C5LM2 and Mia Paca2 tumors received warfarin therapy for 6 weeks until sacrifice. AsPc1-bearing mice received 4 weeks of therapy. Pan02 bearing mice received 3 weeks of therapy. All animals were sacrificed when control animals started to be moribund. Differences in aggressiveness and growth rate between the models was the reasons for the varying treatment lengths in the different models.

Dosing of warfarin

Yanagita et al have shown that warfarin can inhibit GAS6-mediated inhibition of AXL at concentrations below those necessary for affecting the coagulation cascade.³³⁴ In this paper the researchers administered warfarin in drinking water to rats at 0,25-0,5 mg/ml. this gave corresponding serum concentrations of 0,28-1,23 $\mu\text{mol/L}$. No corresponding anemia, increased bleeding tendency or prolongation of prothrombin time were observed. In **Paper I**, we administered warfarin in the drinking water with 1mg/L for immunocompromised mice and 0,5 mg/ml in immunocompetent mice. The rationale for different dosing was an observed toxicity in the immunocompetent mice during pilot experiments. We aimed for a dosing with no anticoagulative effect, and no bleeding complications were observed during the experiments.

4.3.2 Measurements of primary tumor burden and metastases

The primary tumor burden was measured by weighing pancreas and tumor en block. Metastases were macroscopically counted by visual inspection of liver, diaphragm peritoneal surfaces and the abdominal cavity. Metastatic burden was further confirmed with H&E staining of liver sections.

4.4 Induction of EMT

In **Paper I**, we evaluated the relationship between EMT and AXL, and how AXL expression and inhibition would influence on this process. To establish conditions mimicking EMT, cells were grown on chamber slides coated with collagen, and with addition of TGF- β to the media. This is an established method to induce EMT in artificial environments, and is confirmed by an upregulation of vimentin and a downregulation of E-cadherin.³³⁵

4.5 Register study

In **Paper II** we performed a register study taking advantage of two of the major health registries established in Norway. By using the Norwegian identification number, it was possible to couple a cohort from the Norwegian National registry with the Cancer

Registry of Norway and the Norwegian prescription database at an individual level. To be able to perform the coupling we obtained approval from the following instances, in addition to the registries in question:

Regional Committees for Medical and Health Research Ethics, The Data protection official for research at University of Bergen and the Norwegian Data Protection Authority.

4.5.1 The coupling process of different registries

The coupling of information is possible because of the national identification number. The process requires coordination between different instances, in respect of making the process as quick and smooth as possible. NorPD is a so-called pseudonymous registry. Pseudonymization is the situation where the normal person identifier such as name or personal identification number, is replaced with a pseudonym. This pseudonym is unique for each individual, but will not have any relation to the original identifier of the person.³³⁶ Pseudonyms can be used as personal identification in the coupling process. Because the process included the NorPD, the coupling process had to end and be administered by this registry. Figure 10 illustrates the data collection, and coupling of data between the different instances.

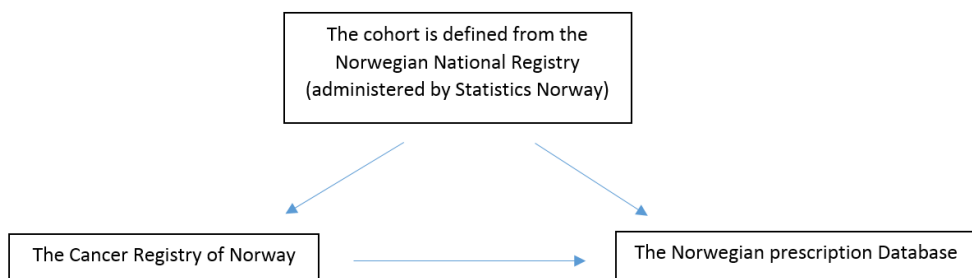


Figure 10. Illustration of the coupling process.

4.6 Statistics

For **Paper I**, statistical analyses were performed using the software GraphPad Prism (GraphPad Prism version 4.00 for windows). Results were expressed as mean \pm s.e.m. of s.d. Data were analyzed by t-test or ANOVA and results were considered significant at $p < 0.05$.

For **Paper II**, statistical analyses were performed using the software STATA IC 13.1 and STATA IC 14.

IRRs were calculated by the method of Mantel-Haenzsel, and adjusted for sex and age. Observed IRRs was considered statistically significant if CI did not include 1.

5. Discussion

In **Paper I**, we evaluated the role of the RTK AXL in PDAC development and metastasis. Our findings in this paper supports the hypothesis of AXL being an important driver of metastatic formation and cancer progression. Inhibition of AXL leads to decreased expression of EMT markers, which further supports the theory of AXL being important for the metastatic processes in cancer. Our findings in **Paper II** supports the preclinical findings of the role of AXL in cancer, and shows that AXL also is important in the cancer initiation process, as well as in more advanced stages of the disease.

5.1 The role of AXL in the development and metastasis of pancreatic ductal adenocarcinoma

PDAC is one of the most lethal of all cancer forms, with no effective treatment regimen. Any research that can contribute to further developments in the treatment of pancreatic cancer is highly appreciated. The receptor tyrosine kinase AXL is associated with many different cancers, including pancreatic cancer, where AXL overexpression is correlated with reduced overall survival and worsened prognosis.²⁴⁴ In the literature, AXL has been strongly associated with increased invasiveness, and metastatic formation.^{118,188,337} This is reported also in pancreatic cancer, both from tumor samples, and in cell culture experiments.²⁴³ AXL inhibition has been suggested as a potential treatment option, and different strategies to achieve AXL inhibition includes development of small molecule AXL inhibitors, antibody-neutralization of GAS6, the ligand of AXL, and as we performed in **Paper I**, inhibition of γ -carboxylation of GAS6, with a subsequent AXL inhibition.^{157,338} Previous in vitro experiments have demonstrated that warfarin strongly inhibits γ -carboxylation of the Gla-domain of GAS6, with a subsequent diminished receptor activation.¹¹⁵ Interestingly, the warfarin doses needed for inhibition of γ -carboxylation of the Gla-domain on GAS6, are shown to be at levels beneath those needed for anticoagulation.²⁷²

In **Paper I**, we demonstrated in vitro that warfarin, as expected, inhibited AXL signaling, and that this inhibition could be repealed when vitamin K was added to the media. This demonstrates that the AXL-inhibitory effect of warfarin are through the depletion of Vitamin K. We also observed a decreased expression of GAS6 and γ -carboxyglutamyl when the cells were treated with warfarin, which further confirmed the mechanisms of action (**Paper I**, Figure 2 A-B). The levels of active phosphorylated AXL were significantly reduced after warfarin treatment, and similarly with treatment with the AXL inhibitor BGB324. Nevertheless, warfarin treatment did not influence on the levels of total AXL in the cells (**Paper I**, Figure 2 C). We also observed a decreased signaling in phosphorylated AKT downstream of AXL after warfarin treatment, confirming a reduced AXL-mediated signal transduction (Paper I, Figure 2 D-E). We further observed a rescue effect of pAKT after addition of GAS6. Knowing that AXL expression is correlated with increased migration^{158,286}, we conducted a migration assay, showing reduced levels of migration in the warfarin treated cells, but only in the AXL expressing cell lines. In Capan-1 cells, which do not express AXL, we observed no difference in migratory capacity (**Paper I**, Figure 2 F). Altogether these findings confirm that AXL expression is important in pancreatic cancer, and that warfarin mediated AXL inhibition will reduce expression of both phosphorylated AXL and its ligand GAS6, which will diminish the migratory and invasive capacity of the cells.

Furthermore, we demonstrated in **Paper I** that warfarin-mediated AXL inhibition reduced metastatic formation in vivo, in 5 different murine PDAC models. (**Paper I**, Figure 1B) In a syngeneic and a genetically engineered mouse model we also observed a statistically significant effect in primary tumor growth (**Paper I**, Figure 1A). The findings were confirmed, observing similar results using an AXL-knockdown cell line, and by selective AXL inhibition by 10C9, an AXL antibody (**Paper I**, Figure 1 F-G). We also observed a reduction in the colony-formation ability of Mia PaCa-2 cells grown in the presence of warfarin (**Paper I**, Figure 3 A-C). The abilities of a cell to grow surface-independent and form colonies are important features of a cancer cell.³³⁹ Our findings was in line with previously published material regarding the ability of anchorage

independent growth after AXL-inhibition.²⁴³ Summarized, our findings establishes AXL as a facilitator for the metastatic potential of pancreatic cancer. Pancreatic cancer has a high level of metastatic formation, and it has been proposed that inhibition of the metastatic process has a great potential in extending life expectancy.³⁴⁰ The results from **Paper I** suggest warfarin-mediated AXL inhibition as a possible treatment option in pancreatic cancer. We also observed an increased reduction in metastatic formation when warfarin was administered together with the established treatment-option, gemcitabine (**Paper I**, Figure 3 F-I). The enhanced effect of established treatment corresponds to what has earlier been described in other cancers, like synergistic effects of AXL-inhibition and cisplatin in the suppression of liver metastases in breast cancer, and increased cell death in AXL-knockdown cells after treatment with cisplatin, carboplatin or doxorubicin in lung carcinoma.^{157,233} The observed additive effect of warfarin and gemcitabine in **Paper I** increases the potential of warfarin-treatment in a clinical setting of pancreatic cancer.

In **Paper I**, but also from other groups, it was demonstrated that AXL-inhibition and subsequent anti-cancer effects of warfarin could be achieved when administered in doses not affecting the coagulation system.^{272,334} VKDPs located outside the liver are shown to be under-carboxylated in the adult population, leaving this population with a sub-clinical Vitamin-K deficiency.²⁹³ Additionally, it is shown in the liver that another enzyme, NAD(P)H-dependent quinone reductase, can function as an alternative facilitator for the recovering of vitamin K from the epoxide metabolite, when VKOR is inhibited by warfarin.²⁹¹ Thus, low doses of warfarin will be able to inhibit VKDPs in peripheral tissues where the steady state concentration of vitamin K is low, while the coagulation-related VKDPs in the liver still will be carboxylated, retaining their biological activity. This finding also supports the theory that the cancer protective effect of warfarin is not due to anticoagulation effects. The possibility of warfarin administration in a low-dose manner is a great advantage for the potential use of the drug in an anti-cancer setting. The ability of using the drug without unwanted side effects from the coagulations system makes it more suitable as a treatment option for cancer patients.

In **Paper I**, we demonstrated a reduction in metastatic growth in all the investigated mouse models. However, we also observed an inhibition of primary tumor growth in the syngeneic and genetic models. (**Paper I**, Figure 1A). These models have, in contrast to the orthotopic models, an intact immune system. In normal physiology, AXL is important in moderating effects of the immune system and preventing auto-immune reactions.¹⁵⁶ When AXL is inhibited, the anti-cancer effects are effectuated via two systems, both direct tumor effects and indirect immunological effects because of repealed AXL-mediated inhibition of the immune system. (Figure 11).

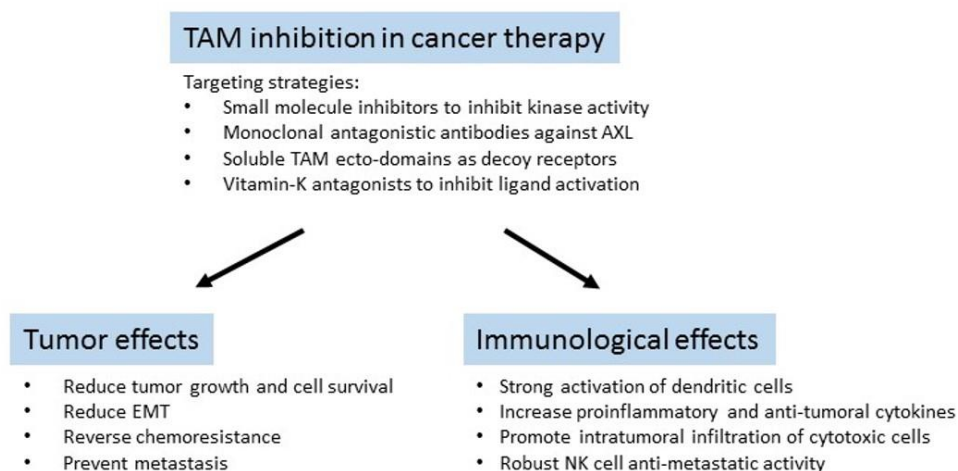


Figure 11: TAM signaling in cancer therapy. Adapted from ²⁸⁴

Park et al, and later Paolino et al, demonstrated an increased cytotoxic ability of NK-cells, after AXL inhibition.^{272,341} However in a recent paper, the researchers argue that AXL signaling will increase effector function of NK cells, so this remains still debated.³⁴² In the model systems in **Paper I**, the orthotopic models will only benefit from the direct tumor effects seen in figure 11, as these mice are not immunocompetent. The syngeneic and genetically engineered models will benefit both from the direct tumor effects, but

also from immunological effects on the tumor. This could explain the observed effects also on primary tumors in these models.

5.2 The role of EMT in warfarin-mediated AXL-inhibition in pancreatic cancer

It has been thoroughly documented that AXL is closely related to the process of EMT.^{65,286,343} Known EMT-inducers, such as TWIST, will upregulate AXL. At the same time, it is shown in pancreatic cancer, that AXL expression contributes to maintenance of the levels of EMT-inducers such as SNAIL, SLUG and TWIST.^{157,243} This is suggesting a positive feedback loop, where EMT-inducers upregulate AXL, and AXL at the same time is maintaining the levels of the inducers. We hypothesized that the observed effect of warfarin-mediated AXL-inhibition in pancreatic cancer in **Paper I**, was related to an inhibition of the EMT process. A blocking of EMT is shown to decrease the metastatic potential and the migratory ability of a developing tumor, in a wide range of tumors, including pancreatic cancers.^{344,345} However, a report from 2015 claims that EMT suppression will not limit the metastatic development in pancreatic cancer. In a mouse model with deletion of the EMT-inducers SNAIL or TWIST, the group observed no change in the metastatic potential of the tumors.³⁴⁶ Previously, it has also been a challenge to find consistent evidence of upregulation of mesenchymal markers, and downregulation of epithelial markers in this setting, with a high variability of expression between tumors.³⁴⁷ An explanation of this variability is the theory that not all cancer cells undergo EMT at the same time, resulting in cells within a certain tumor presenting with different levels of EMT at a given time-point. According to this theory, only a small fraction of the cells in a certain tumor will have gained mesenchymal properties and are presenting with detectable mesenchymal markers as evidence for EMT.^{96,344}

In **Paper I**, we investigated the relationship between EMT and AXL expression in pancreatic cancer. We found, as expected, that EMT induction by collagen and TGF β , gave an increase in AXL phosphorylation, and also increased expression of ZEB1 and nuclear β -catenin, and that this effect was blocked when warfarin was added. We also experienced increased levels of the mesenchymal markers Vimentin and ZEB1 with

addition of GAS6 to AXL-expressing cells. Furthermore, we observed a downregulation of the mesenchymal marker Vimentin, and a simultaneous increase in the epithelial marker E-cadherin after warfarin treatment (**Paper I**, Figure 4 A-C). These results support previous reports about EMT in pancreatic cancer³⁴⁸ and contributes with evidence to the theory that EMT is a strong regulator of the invasive and metastatic potential of pancreatic tumors.

5.3 Vitamin-K in cancer

Warfarin is inhibiting AXL by inhibiting the conversion of oxidized vitamin K to the active reduced vitamin K. This process is catalyzed by the enzyme VKOR. When this enzyme is inhibited, there will be a depletion of active vitamin K in the body (See section 1.6, figure 9). Active vitamin K is a necessary substrate for the GGCX enzyme, which converts glutamate to γ -carboxyglutamate at the Gla-domain of VKDPs. This conversion is necessary for the biological function of VKDPs.³⁴⁹ This has been demonstrated for AXL-GAS6 specifically, where γ -carboxylation of GAS6 is shown to be required for ligand-induced receptor activation.^{110,132,350} Non-carboxylated GAS6 retains full AXL-binding affinity, but is not capable of inducing receptor activation.²⁸³ This is converting the ligand into an AXL antagonist. Interestingly, upregulation of GGCX is observed together with upregulation of AXL/GAS6 in several different cancers, facilitating a high receptor activity.²⁸³

Our findings from **paper I** and **paper II** proposes a cancer-promoting relationship between vitamin K and cancer, related to γ -carboxylation of GAS6 and subsequent AXL-activation. Nevertheless, there are some contradictory findings regarding Vitamin K and its role in cancer development. Some studies has shown that intake of menaquinon-4 (MK-4), an isoform of Vitamin-K, could have a cancer protective effect, especially in liver cancer.²⁹¹ The mechanisms behind this effect are poorly understood, without any defined molecular target, although an impact of cell cycle has been proposed.³⁵¹ MK-4 constitutes only a small fraction of dietary vitamin K, and it is not likely that the proposed cancer protective effect would have any impact of the results from **Paper II**. In that case,

it would give rise to more cancers in the warfarin treated group, as MK-4 also would be inhibited by warfarin.

5.4 Warfarin use and cancer incidence

Based on our findings from **Paper I**, we asked the question if warfarin could influence on cancer incidence in humans. To address this we performed a register based cohort study, using data from the comprehensive Norwegian health registries. The free, universal health care system in Norway ensures that these registries have broad coverage and are well suited to be basis for a population study. The vitamin-K antagonist warfarin is a drug that is commonly administered in the adult population, most prevalent over 50 years of age, and as described in the introduction, with increasing prevalence with increasing age.²⁹⁹

The most widespread effect of increased *AXL* expression is related to cancer progression and metastatic dissemination. *AXL* overexpression could also be important in the process of tumor initiation. Asiedu et al published in 2014 results demonstrating that *AXL* expressing cells is more tumorigenic than *AXL*-negative cells.²⁸⁶ This is in line with demonstrated effects on tumorigenesis also in other cancers, such as pancreatic cancer, prostate cancer and glioblastoma.^{103,263,352} After primary tumor is established, effects on tumor growth have been demonstrated in many tumor forms, such as PDACs and glioblastomas. In other tumors, primary tumor growth is less or not affected.³⁵³

Several reports have been published on warfarin use and cancer, both clinical trials and register-based studies. The results have been somewhat conflicting. In 2011, Pengo et al published a report assessing the effect of warfarin treatment on cancer incidence and survival in a cohort of 76,008 persons. They found a Hazard ratio (HR) of newly diagnosed cancer of any type in the exposed group of 0.88, and a HR of 0.69 in prostate cancer.³⁵⁴ These findings correspond to our findings in **Paper II**, with an IRR of 0.84 in all-site cancer, and 0.69 in prostate cancer (**Paper II**, Table 2). Contradictory, Kinnunen et al evaluated the risk of prostate cancer in warfarin users in a Finnish population of

78,615 men. In this study they reported an elevated risk of prostate cancer in warfarin users compared to non-users, with an adjusted HR of 1.11.³⁵⁵ To define warfarin users, they took into consideration any use of warfarin prior to cancer diagnosis, also prescriptions made shortly before diagnosis, when it is likely that the cancer was already established. This could be a source to overestimation of cancer cases in the user group. Due to the wide definition of a warfarin user, they define 16.7 % of their population as warfarin users, compared to 7.4 % in the population in our study (**Paper II**, Table I). An estimate of 16.7 % users in the investigated population is high also considering the prevalence in the general population, which has been reported to be approximately 3 % in a general population, and 9 % in a population older than 65 years.²⁹⁸

The opportunity to perform a register based population study, with a large cohort gives a unique possibility to investigate the question of anti-cancer effects of warfarin in a large scale. One of the major limitations of a study based on the major health registries in Norway is the lack of information of possible confounding factors in the material. The available information includes demographic information, and information extracted from the registries in question, the CRN and NorPD. The registries do not contain any information regarding lifestyle factors as food intake, physical activity, smoking or alcohol use. These are factors that potentially could influence on the risk of cancer development. However, the properties of broad-covering registries allow inclusion of a great number of individuals, which makes it possible to perform analyses, also on rarely occurring cancer forms.³⁵⁶ This is a major advantage with large cohorts, and it has to be considered together with the concurrent limitation of lack of information on life style factors.

In **Paper II**, we observed an overall cancer protective effect, although the effect was not consistent for every included cancer site in the material. We observed that our findings corresponds to cancers with a known close relation to AXL, such as lung, prostate and breast.^{157,234,263} In **Paper II**, table 2, we see that there is no significant warfarin protective effect for overall Bone-marrow related cancer, or leukemia as a group of diseases.

Nevertheless, for AML, we observe a significant IRR of 0.82. Up-regulation of AXL is shown to be closely related to a worsened prognosis and poor survival in AML.²¹⁸ AXL upregulation is shown in a much lesser extent in the lymphoid leukaemias.²²⁰ Based on the hypothesis of the warfarin-AXL relationship, we did not expect a major reduction of the incidence of lymphatic leukaemias after warfarin treatment. Consistent with this, in the results from **Paper II**, we observe a cancer-protective effect of warfarin in AML, and on the other hand, there is no significant difference in the incidence of CLL in warfarin users compared to non-users. (**Paper II**, Table 2).

It is known that thromboembolic disease, such as venous thromboembolism and pulmonary embolism, is associated with an elevated cancer risk. The risk is highest the first year after diagnosis, but an increased risk has been shown also later.³⁵⁷ This was an important confounding factor in our study, which needed to be addressed. Thus, we also evaluated the cancer risk in a subgroup of the warfarin-users, which were prescribed warfarin after atrial fibrillation or flutter. This group lacked the preexisting occult malignancy risk of the thromboembolic patients. In this group, we observed an overall IRR in the users of 0.62 (**Paper II**, Supplementary Table 2). This reduction in IRR was expected, and in line with the knowledge of an increased cancer risk in the thromboembolic patients.

5.6 Warfarin in the era of Non-vitamin K anticoagulants

In the recent years, a new class of anticoagulant drugs has emerged, so-called non-vitamin K anticoagulants (NOACs). The drug-class consists of four different drugs. Dabigatran is targeting thrombin, Apixiban, Edoxaban and Rivaroxaban are targeting factor Xa.^{358,359} The use of NOACs as a replacement for warfarin has some important advantages. NOACs do not require regularly laboratory monitoring, as the therapeutic window has a wide range. There are also fewer interactions between food and other drugs, which gives a more stable bioavailability, with reduced risk of being outside the therapeutic range.³⁶⁰ At the same time, NOACs are shown to be as effective, or even superior to warfarin in reducing the risk of thromboembolic events after atrial fibrillation. The risk of side effects are similar to warfarin, with no difference regarding major bleedings, but with an increase in gastrointestinal bleedings.³⁶¹ In **Paper II**, we propose a cancer protective effect of warfarin, with a 16 % lower risk of all-site cancer in the users compared to non-users. For selected cancer sites, the observed protective effect is further lowered, with a 20% lower IRR in lung cancer and 31% lower IRR in prostate cancer compared to controls. (**Paper II**, table 2) Based on the results from **Paper II**, the switch from warfarin to other anticoagulation regimen could have the potential to give an increase in cancer incidence, affecting millions of patients on a world basis. The loss of a specific cancer protective effect of warfarin should be taken into consideration when the decision of anticoagulant regimen is to be made.

6. Concluding remarks

Based on our findings, we claim that the RTK AXL is an important facilitator for pancreatic cancer metastatic dissemination and outcome. Warfarin-mediated AXL-inhibition is shown in vivo to effectively prevent or decrease metastatic formation in different model systems. We also show that the observed cancer-protective effect is achieved when warfarin is administered in doses not affecting the coagulation system. We show that AXL expression is strongly related to the EMT process in pancreatic cancer, and that warfarin mediated AXL-inhibition will reverse the EMT process, and increase the expression of epithelial markers, and recover epithelial properties in the cells.

In a register-based cohort study, we also show that warfarin-use is associated with lowered cancer incidence in a large Norwegian cohort. The cancer protective effect of warfarin is observed both for overall cancer, and also for several cancer sub-sites. We hypothesize that the observed association is due to warfarin-mediated AXL-inhibition in the cancer cells. Our findings in **Paper II** show a broad cancer protective effect of an old, commonly used drug. Our findings from **Paper I**, suggests that it would be possible to obtain warfarin-mediated cancer protection also in low doses, not affecting the coagulation system. These findings should be taken into consideration in the era of NOACs, when the choice of anticoagulant therapy is to be made. Further studies are needed to enlighten the possibility of using warfarin in an anti-cancer setting.

7. Future perspectives

The RTK AXL is known to have important roles in normal physiology, mostly related to hemostasis, phagocytosis of apoptotic cells, and dampening of the immune system. Collectively, the role of the receptor appears to be related to maintenance of body homeostasis, such as resolving inflammation response. The role of AXL in cancer is somewhat paradoxical to this pattern. In cancer, AXL signaling lead to increased stress, for example through enhanced invasiveness, migration and prolonged cell survival, even of highly atypical cells. The relevance of AXL's role in normal physiology as applied to the cancer setting is not very well described. It seems that AXL-mediated contributions to normal tissue homeostasis are effectively co-opted by cancer cells to mitigate stress during tumorigenesis. We know that cancer-related AXL-effects are associated with AXL upregulation, and very rarely are the result of mutations. One hypothesis is that the pro-tumor effects of AXL require a certain degree of dynamic expression. Indeed a recent paper describes the existence of rare tumor cells that sporadically express high AXL levels ("AXL jackpot cells") that are the source of resistant colonies.³⁶² Importantly, these cells are not genetically fixed but rather transition into an AXL-expression state only when necessary. Future research on this conversion would be of interest, to clarify how the levels of AXL reflect changes in the surrounding microenvironment.

We have also established the role of warfarin as an AXL-inhibitor. This 70-year old drug is widely used as an anticoagulant, but has on occasions been proposed as an anti-cancer drug. Based on the results from this thesis we propose a potential use for warfarin in the anti-cancer setting. The possibility to use the drug at sub-coagulation doses further strengthens the potential for use as cancer prevention. Further experiments, to elucidate the potential benefits of the drug would be of great interest. In our register-material we have the possibility to look into cancer-specific survival in warfarin users compared to non-users, and this is a natural step following the experiments of this thesis. Furthermore, after the implementation of NOACs as

alternative anticoagulation regimen, it will be possible to perform a register study that compares cancer incidence in warfarin users versus users of NOACs. This could more definitively differentiate the coagulation-independent anti-cancer properties of warfarin, and a study with this focus is in planning.

We have hypothesized that warfarin have an effect in the tumor initiation phase. To take advantage of this effect clinically, it would be necessary to start warfarin treatment before the start of cancer development. This would imply a widespread treatment of a healthy population, and could be a challenge both ethically, logistically, and from a socioeconomical perspective. Nevertheless, it could be interesting to administer the drug in an adjuvant setting, to exploit the proposed effect of tumor initiation to prevent recurrent disease.

Overall, further defining the role of AXL in cancer development and the cancer protective effects of AXL inhibition are warranted. Future steps would be both on a pre-clinical and register-based level, but also eventually translation into clinical trials to establish the overall cancer-protective potential for using warfarin-mediated AXL-inhibition in a clinical setting.

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